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Authentication and investigation of potential hepatotoxicity of Black Cohosh

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Abstract

Black Cohosh (*Actaea racemosa*) is one of the highest selling medicinal plants, ranking as the sixth best seller in the US in 2015 (Smith et al., 2016). However, this popularity has been tarnished by claims of hepatotoxicity. The investigation of these reports has determined that implicated products did not contain Black Cohosh plant material. Other reports were shown to be incomplete or had other factors contributing. This has led to the suspicion that cases of adverse reactions may in fact be linked to cases of substitution or adulterations with Asian species of *Actaea*, rather than to *A. racemosa.* (Jordan et al., 2010). This shows the need for authentication of Black Cohosh products.

In this study various DNA based authentication methods were developed. The first, PlantID is capable of discriminating between *Actaea racemosa* and four potential adulterant species; *Actaea cimicifuga, Actaea cordifolia, Actaea podocarpa and Caulophyllum thalictroides,* in a single PCR reaction. The resulting fragments are scrutinized using gel electrophoresis. Other platforms of analysis were trialled with little success. The second was a qPCR based method. These assays are competent in detecting *A. racemosa*, *A. cimicifuga* and *A. dahurica* species and are compared to a generic primer capable of amplification of ten *Actaea* species. This enables the user to detect specific species in comparison to how much *Actaea* species are present as a whole. This assay was extensively tested on many materials and products available in the UK and the USA. Out of 34 products assessed it was possible to extract DNA from 32. From the UK market it was found that five products contained undeclared species. From the US market it was found that six products contained undeclared species. All of the THR registered products were found to contain only the authentic species *Actaea racemosa.* This was a reassuring result from the analysis and adds further value to the scheme of THR.

Sequence data from GenBank was used to assist in assigning species to sequenced DNA samples. The data contained on GenBank was scrutinised using various bioinformatics tools. Sequences were organised into molecular taxonomic units using tree diagram software. This showed efficiently and

visually which sequence entries were reliable to use based upon grouping. This analysis showed that the nuclear internal transcribed spacer (nrITS) was an ideal barcoding region and that maturase K (MatK) was a poor choice for *Actaea* species.

To address the issue of hepatotoxicity claims, cultured human hepatocyte derived cells were treated with 60% ethanol extracts of *Actaea racemosa* and Asian *Actaea*. A qPCR array was utilised to assess 84 genes associated with hepatotoxicity across various concentrations of extract. The collective array output gave a plethora of data which was analysed using bespoke online software from the manufacturer. Stringent quality controls were included on the arrays which gave confidence of results. There were small changes noted for *Actaea racemosa* and some activity for the Asian *Actaea* treated cells was also seen. An LDH and MTT assay were used to assess cell viability and toxicity in two human hepatocyte derived cell lines. *Actaea racemosa* showed no significant effects whereas the Asian *Actaea* extract showed a notable decrease in cell viability and significant release of LDH indicating toxicity. The Asian *Actaea* material used to manufacture extracts was of questionable species origin but determined to be either *A. dahurica* or *A. cimicifuga.* The results from these experiments were unfortunately not as conclusive as hoped, but did show some evidence of a more likely culprit of toxicity originating from Asian *Actaea* species.

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Tables

Abbreviations

AFLP - Amplified Fragment Length Polymorphisms

AHPA – American Herbal Products Association

ALP - Alkaline Phosphatase

ALT - Alanine Amino Transferase

APAP - Acetyl-para-aminophenol

ARMS – Amplified Refractory Mutation System

AVG - Average

bar-HRM - Barcode High Resolution Melting

BC - Black Cohosh

BGBM – Botanical Garden and Botanical Museum (Berlin)

BHMA - British Herbal Medicine Association

BLAST - Basic Local Allignment Search Tool

BNO - Bionorica

BOLD - Barcode of Life Database

BP – British Pharmacopoeia

CAMAG - Chemie-Erzeugnisse & Adsorptionstechnik AG

CBOL – Consortium for the Barcode of Life

cDNA - Complementary Deoxyribonucleic Acid

CITES – Convention on International Trade in Endangered Species of Wild Fauna and Flora

CMV - Cytomegalovirus

CPC - Chinese Pharmacopoeia Commission

Ct - Threshold cycle

DEPC – Diethyl Pyrocarbonate

DHU - Deutsche Homöopathie-Union

DILI – Drug Induced Liver Injury

DNA - Deoxyribonucleic Acid

dNTPs - Deoxynucleotide Triphosphates

dPCR - Digital Polymerase Chain Reaction

DSHEA – Dietary Supplement Health and Education Act 1994

DSI EC - Dietary Supplements Information Expert Committee

EBV - Epstein-Barr Virus

EDQM – European Directorate for the Quality of Medicines

EDTA - Ethylenediaminetetraacetic acid

EMA - Economically Motivated Adulteration

EU - European Union

FDA – US Food and Drug Administration

GABA - Gamma Aminobutyric Acid

GC - Gas Chromatography

GnRH – Gonadotropin Releasing Hormone

HAV - Hepatitis A Virus

HBV - Hepatitis B Virus

HCl - Hydrochloric Acid

HCV - Hepatitis C Virus

HPLC – High Performance Liquid Chromatography

HPLC-ELSD - High Performance Chromatography-Evaporative Light Scattering Detection

HPTLC – High Performance Thin Layer Chromatography

HRM – High Resolution Melting

HRS – Herbal Reference Standards

HRT – Hormone Replacement Therapy

HSV - Herpes Simplex Virus

IESPB – Illinois Endangered Species Protection Board

INSDC - The International Nucleotide sequencing Database Collaborative

ITS - Internal Transcribed Spacer

IUCN - International Union for Conservation of Nature and Natural Resources

KMI – Kupperman Menopausal Index

LC - Liquid Chromatography

LC-MS - Liquid Chromatography Mass Spectrometry

LDA - Linear Discrimination Analysis

LDH – Lactate Dehydrogenase

LH – Luteinising Hormone

matK - Maturase K

MgCl2 - Magnesium Chloride

MHRA – Medicines Healthcare Regulatory Authority

MOTU – Molecular Operational Taxonomic Unit

MRS – Menopause Rating Scale

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUSCLE - Multiple Sequence Comparison by Log-Expectation

NCBI - National Center for Biotechnology Information

NCCAM - National Centre for Complementary and Alternative Medicine

NGS - Next Generation Sequencing

NHESP – National Heritage Endangered Species Programme

NIH – National Institute of Health

NMR - Nuclear Magnetic Resonance

nrITS - Nuclear Internal Transcribed Spacer

ODS - Office of Dietary Supplements

PCA - Principle Component Analysis

PCR – Polymerase Chain Reaction

PGM - Personal Genome Machine

Ph.Eur – European Pharmacopoeia

PhyML - Phylogeny estimation using Maximum Likelihood

PMDA - Pharmaceutical and Medical Devices Agency

PPC - Positive PCR Control

PPRC - The Pharmacopoeia of the People's Republic of China

qPCR - Quantitative Polymerase Chain Reaction

RAPD – Random Amplified Polymorphic DNA (Deoxyribonucleic Acid)

RBG – Royal Botanical Gardens (Kew)

RFLP – Restriction Fragment Length Polymorphism

RNA - Ribonucleic Acid

RTC - Reverse Transcription Control

SCAR - Sequence Characterized Amplified Region

SSRs - Simple Sequence Repeats

STRs – Short Tandem Repeats

TBE - Tris Borate EDTA Buffer

TFDA – Taiwan Food and Drug Administration

THM – Traditional Herbal Medicine

THMPD – Traditional Herbal Medicinal Products Directive

THR - Traditional Herbal Registration

TLC – Thin Layer Chromotography

UPLC-QTOF-MS - Ultra performance liquid chromatography-quadrupole orthogonal time of flight-mass spectrometry

UPS – United Plant Savers

USDA - United States Department of Agriculture

USFWS - US Fish and Wildlife Service

USPC - United States Pharmacopoeia Committee

USP-NF - United States Pharmacopoeia and National Formulary

UV - Ultra Violet

VZV - Varicella Zoster Virus

WHO – World Health Organisation

1 Introduction

1.1 Medicinal Plants

The use of medicinal plants can be traced back thousands of years. Evidence has been found in Ancient Egyptian papyri, e.g. Ebers papyrus (c.1550 BC) and in texts detailing the use of medicinal plants dating back as far as 400BC in the *Corpus Hipocraticum* authored by Hippocrates (460-370 BC). The next earliest text is from 300BC with Theophrastus of Eresos authoring *Enquiry into Plants* (371-287 BC). Better known ancient texts include Dioscorides' *De Materia Medica* (40-90 BC) *and* Galens' (129-210 AD) *De Simplicium Medicamentorum Facultatibus Libr XI* (1561) (Leonti and Verpoorte, 2017). Medicinal plant usage has been found to date back centuries all over the globe including China, India and Tibet (Schwabl and Vennos, 2015). Today this inherited knowledge is known as Traditional herbal medicine (THM), and includes systems such as Ayurvedic and Traditional Chinese Medicine. Over time, the use of some systems of THM has declined due in part to the introduction of preventative medicine such as vaccines, by the use of antibiotics to treat infectious disease and the development of organic chemistry which has allowed synthesis of new effective medicines. Natural medicines are however still heavily relied upon in developing countries with up to 80% of the world still using them for their primary health care needs. The cost or access to modern medicine and distrust are the main factors (Mishra et al., 2016). With advances in technology and understanding there is the potential to provide evidence of the efficacy of these medicinal plants and due to this their use is on the rise again (Leonti and Verpoorte, 2017). However, with the increased use of THM comes the increased need for these products to be regulated, to ensure that safe products are on the market. In the UK and Europe, as per the Traditional Herbal Medicinal Product Directive (Directive 2004/24/EC), herbal medicines are regulated by Traditional Herbal Registration (THR). Registration does not prove that a herbal medicine has efficacy but gives assurance of safety, quality, good manufacturing practices, traditional usage and the provision of

appropriate information. So although efficacy is not proven with clinical data, it is implied with a history of use. This was lacking prior to the registration scheme in the UK. This registration arose to promote harmonisation of product quality in the UK with other countries of the European Union and to address safety concerns. In the US, herbal medicines are regulated as Dietary supplements, by the FDA as part of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Pharmacovigilance is the assessment, understanding, detection and prevention of adverse effects or associated problems of any given drug. Pharmocovigilance of herbal medicines is and remains a challenging area due to several factors. When demand of herbal drugs exceeds supply, the risk of adulteration or substitution significantly increases. The danger in these cases is that the authentic species is replaced with one that has a different effect or no efficacy for the intended use. The replacement or adulterant species could also be harmful to the recipient such as the case with *Stephania* based medicines adulterated with *Aristolochia* which has found to be nephrotoxic and even carcinogenic (Tankeu et al., 2016). In TCM the plants have very similar names with *Staphania* being called *Han Fang Ji* and *Aristolochia* being called *Guang Fang Ji.* Registered products must be produced with authentic species and be free from heavy metals and other undeclared pharmaceutical agents. They must also not contain any species from the 'Banned and Restricted Herbal Ingredients' list provided by the Medicines Healthcare Regulatory Agency (MHRA). Standardisation between products is also an issue which registration will help to overcome (Barber, 2014).

In Europe, the use of plants for human medicinal purposes is regulated by the European Union (EU) with the Traditional Herbal Medicinal Product Directive (THMPD) (Directive 2004/24/EC). This requires evidence of a plant's traditional use for 30 years within the EU. For a plant medicine from outside of the EU, it has to be shown to have been used within the EU for 15 years and 15 years previously elsewhere (Cranz and Anquez-Traxler, 2014).

The THMPD also has a strict definition for a medicinal herbal product, which avoids confusion with 'food supplements'. Food supplements can be derived from herbal products and have a beneficial effect on health but do not actually treat human disease as outlined in the definition below.

A food supplement is defined as;

"Any food the purpose of which is to supplement the normal diet and which –

a) Is a concentrated source of a vitamin or mineral or other substance with a nutritional or physiological effect, alone or in combination; and

b) Is sold in dose form"

A medicinal herbal product is defined as;

"A product presented for treating or preventing disease, or which may be administered with a view to restoring, correcting or modifying physiological function in humans, falls within the definition of a medicinal product and is subject to the requirements of the Medicines Directive".

The directive came into force in 2004. New products have to comply immediately and existing products had until May 2011 to be registered. The process of registration, which shows compliance with the directive, is both expensive and time consuming. It is hoped that the THMD will improve the quality of products on the market (Vlietinck et al., 2009).

1.2 The British Pharmacopoeia (BP) and the European Pharmacopoeia (Ph. Eur.)

The creation of a Pharmacopoeia in the UK dates back to 1618 with the London pharmacopoeia. This text was published by the Royal College of Physicians in Latin, the then common language in science, as it was aimed for use by physicians and apothecaries and not the common (uneducated) man. The text was proclaimed by King James I to be solely used by apothecaries during the preparation of medicinal products. The Edinburgh Pharmacopoeia was first published in 1699 by the Royal College of Physicians of Edinburgh. The London Pharmacopoeia was used by the Irish up until 1850 when the

first Dublin Pharmacopoeia was produced. The Medical Act 1858 put in place the production of the British Pharmacopoeia which would supersede the London, Dublin and Edinburgh Pharmacopoeia. This was a vastly difficult task and took considerable time; the British Pharmacopoeia was first published in 1864. The text was not popular and was criticised heavily due to its many imperfections. (Cartwright, 2014).

Over the years further editions were published with the publication still causing mass dissatisfaction amongst the professions. In 1929 a new commission was formed chaired by of Arthur Beddard, a consulting physician. The committee was taken in a new direction with regular meetings and the assistance of six sub-committees. The committee appointed Charles Hampshire as secretary, an extremely educated individual who at the time was the chief pharmacist at the University College Hospital with a degree in chemistry and qualified in medicine. The development of the next edition of the pharmacopoeia was supported by The Pharmaceutical Society who set up a laboratory dedicated to work on the pharmacopoeia. The next edition was published in 1932 and heralded as 'the Chemists' Bible'. New editions were published regularly. In 1968, following the disaster of the drug thalidomide, the copyright of the British Pharmacopoeia was transferred to the crown and the commission staff became civil servants of the Department of Health. Thalidomide was a drug that first went on sale in Germany as a sedative. It went on to be sold in 46 countries which resulted in the deformity of 10,000 babies. This disaster was central to shaping the drug regulation system (Rago, 2008). From then on, new editions were published every five years with Addenda regularly released in response to the fast advances of drug development and analytical chemistry. This continued until 1990 when Robin Hutton was appointed secretary and introduced yearly editions of the publication. He also introduced a CD ROM of the text, a first for any pharmacopoeia. Today the committee also provides expertise to the European Pharmacopoeia ensuring harmonisation between the BP and Ph. Eur (Cartwright, 2014).

The creation of the European Pharmacopoeia began in 1964 when Belgium, Germany, France, Luxembourg, Italy, The Netherlands and the UK signed a convention drawn up by the Council of Europe. One of the aims of the Council of Europe was to promote the standard of living for the people of the countries involved, and another was to promote unity between members. With this in mind it was decided that an elaboration of a European Pharmacopoeia was to be made, and for the monographs included to become official standards for pharmaceuticals and raw medicinal products within the member countries (Council of Europe, 1964). The UK continued to use and develop the British Pharmacopoeia incorporating the European Pharmacopoeia where applicable.

In 1989 a Protocol to the Convention was signed which agreed approval by the members and hence the people of these countries (Council of Europe, 1989). This agreement entered into force in 1992. Today it is developed by the European Directorate for the Quality of Medicines and Healthcare (EDQM), and its mission statement is still true to that of the original Convention;

"The mission of the European Pharmacopoeia is to:

- *promote public health by the provision of recognized common standards for use by healthcare professionals and others concerned with the quality of medicines,*
- *facilitate the free movement of medicinal products in Europe,*
- *ensure the quality of medicinal products and their components imported into or exported from Europe,*
- *design European Pharmacopoeia monographs and other texts to be appropriate to the needs of regulatory authorities, those engaged in the quality control of medicinal products and their constituents and to the manufacturers of starting materials and their products"*

(EDQM, 2017b)

There are 37 Members of the Ph. Eur. These include the European Union, the EU member states and European Free Trade Association countries. There are 30 observers including the World Health Organization and The Taiwan Food and Drug Administration (TFDA) of the Ministry of Health and Welfare. The observers are made up of European countries and non-European countries. The Members benefit from being able to participate in decision making regarding technical content whereas Observers can only view the material and participate in the scientific work involved in its development. A full list of Members and Observers can be found below in Table 1.

Table 1: A full list of members and observers of the European Pharmacopoeia (Correct as of August 2017)

Members	
The European Union	
EU Member States	Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, The Netherlands, Portugal, Spain, Sweden and The United Kingdom, Croatia, Turkey, Ukraine, former Yugoslav Republic of Macedonia, Cyprus, The Czech Republic, Estonia, Hungary, Latvia, Lithuania, Malta, Poland, The Slovak Republic, Slovenia, Bulgaria, Bosnia and Herzegovina, Serbia and Montenegro Romania.
European Free Trade Association Countries	Iceland, Norway and Switzerland.
Observers	
The World Health Organization	
The Taiwan Food and Drug Administration (TFDA) of the Ministry of Health and Welfare	
European Countries	Azerbaijan , Albania, Armenia, Republic of Belarus, Georgia, Kazakhstan, Moldova, and The Russian Federation
Non-European Countries	Algeria, Guinea, Argentina, Australia, Brazil, Canada, China, Israel, India, Republic of Korea, Kazakhstan, Madagascar, Malaysia, Morocco, Senegal, Singapore, South Africa, Syria, Tunisia and The United States

Within the EDQM, two working bodies develop the Ph. Eur. The European Pharmacopoeia Commission is comprised of scientists who prepare the monographs. The Public Health Committee of the Council of Europe deals with other administrative aspects of the monographs but does not take part in the technical content (EDQM, 2017b).

1.2.1.1 Monographs

The BP and the Ph. Eur consist of a vast number of monographs. The monographs focused on in this work are the herbal drug, and herbal drug preparation monographs. A monograph is a collection of information about a pharmaceutical which includes standardised methodology for identification, quantification and tests for quality purposes. The monograph begins with the common name of the pharmaceutical, and in the case of herbal drug and herbal drug preparations there may or may not be an English or Latin name included. There is usually a first and second identification. The second identification is usually a simplified version of the first identification but it may contain tests that are also listed in the first identification. This is in the case where a pharmacist may be obliged to test a product but may not have the necessary equipment to carry it out. Macroscopic characteristics are listed for a clear identification, and microscopic characteristics (in the case of a powdered product) are included also. Sometimes diagrams are also incorporated where necessary. Identification techniques may also be used as quality tests or quantification tests. The main method used for identification is Thin Layer Chromatography (TLC), but may be another technique such as gas chromatography (GC) or liquid chromatography (LC) (EDQM, 2017b). The monograph also lists a number of tests for quality purposes. This is outlined in Table 2;

Table 2: A list of typical quality tests included in the Herbal monographs of the European Pharmacopoeia

Test	Application	When it is used
Total Ash	Carried out on powdered drug	Always, unless justified
Ash insoluble in hydrochloric acid	Detects unacceptable levels of certain minerals	Depends upon the nature of the drug
TLC	Detects unwanted plant species	If unwanted plant presence is suspected
GC/LC	Detects unwanted plant species Aids control of evaporation/degradation of substances that must be present	If unwanted plant presence is suspected
Foreign matter	Parts of the plant/adulterant species/minerals not in the definition – this is a limit of 2% unless otherwise stated	Always, unless otherwise stated

Heavy Metals	A general method listed in another section	Used where contamination is suspected or when it is known that a herbal drug accumulates a particular metal
Loss on drying	To certify if the drug has been dried out Herbal drugs that are not dried enough may harbour yeasts or moulds Usually 2 hrs = 10% water loss	Where herbal drugs may be dried.
Water	Determination of water content by distillation	Used when levels of essential oils present exceeds 1%
Swelling index	Used for certain hydrocolloid-containing herbal drugs	
Bitterness value	For herbal drugs containing bitter principles	
Extractable matter		Where no other suitable assay is available
Microbial quality		Limits differ for treated and non-treated material, i.e. Heat.
Pesticide residues		Where specified

adapted from (EDQM, 2017c)

For quantification purposes, assays are listed. This is included at all times where ever feasible. If possible, the method of choice is gas or liquid chromatography, but if there are good quality chemical markers present, spectrophotometry can be used, although this is not encouraged. The EDQM provides information about the sourcing of Herbal Reference standards (HRS). All identification and quantification tests will have reference standards for comparison (EDQM, 2017c).

These monographs are the standards adhered to by members and observers of the Ph. Eur for identification, quantification and overall quality testing of herbal products and other pharmaceuticals (Council of Europe, 1964).

In 2014, the BP committee set up a DNA working group with the aim to develop DNA based identification methods. Where the DNA method for a herbal medicine is not an identification method, the information is provided in a supplementary chapter entitled, SC VII D. DNA Barcoding as a tool for Botanical Identification of Herbal Drugs. The methods are detailed in Appendix XI V. Deoxyribonucleic Acid (DNA) Based Identification Techniques for Herbal Drugs. This advance is independent from the Ph. Eur. The ability of the technique is based on DNA barcoding which is

explained in the next section: 1.3 DNA Barcoding. The barcode region *trnH-psbA* has been used for identification of *Ocimum tenuflorum* and a trnH-*psbA* standard for use in the outlined method has been made available for purchase. For barcode development it is noted that nrITS, *rbcL*, *trnL-F* and *matK* are also being considered for other plant species (BPC, 2017b).

1.3 DNA Barcoding

The Barcoding effort began in 2003 when Dr Paul Hebert, University of Guelph, Canada, proposed the use of DNA barcodes to identify organisms at species level. The idea was to use short gene sequences as a means of identification (CBOL, 2017). Current methods require very skilled and experienced taxonomists to examine the morphology of a specimen in order to identify it, and damaged or immature specimens could be impossible to identify even to these experts. Chemical methods require the identification of chemical markers. These markers can be present in other species and can also be added to a sample falsely to pass as the desired product (Palhares et al., 2015). It is however possible to extract DNA from these specimens including those that may be aged and degraded, so DNA barcoding was considered a possible solution to these problems (Cowan et al., 2006).

The Sloan Foundation awarded The Smithsonian Institution sufficient funds to create a Consortium for the Barcode of Life (CBOL). The main aim of the CBOL is to promote the advance of barcoding. Since the creation of the CBOL, further large sums of money have been awarded to the Smithsonian Institution and major conferences have taken place in the UK, Taiwan and Mexico. The International Barcode of Life Project was initiated in 2010. Scientists from many disciplines all over the world are working together in order to construct a library of references for DNA barcodes of multi-cellular organisms. The first phase of this effort began in 2010 and includes barcoding 500,000 species. There are currently two databases, one of which is the Barcode of Life Database (BOLD) and the other is The International Nucleotide sequencing Database Collaborative (INSDC) (CBOL, 2017).

The ideal barcode for animals was agreed at an early stage to be the cytochrome oxidase 1 gene (COX1). This has been demonstrated to have the ability to distinguish 90% of animals (de Boer et al., 2015). However, the much slower rate of evolution of the COX1 gene in plants has deemed it unsuitable for use as a barcode (Kress et al., 2005).

For plants, several candidate barcodes have been considered, but each proposed region has been shown not to be sufficient when used individually. This led to the conclusion that for plants, combinations of barcode regions would be required to meet the specification of a good barcode

According to Hollingsworth (2009) a good barcode should meet the following criteria;

1. It should be possible to routinely PCR with a single primer pair
2. It must produce good quality data with bidirectional sequencing
3. It should require little manual editing of sequence traces
4. It should have maximum discrimination amongst species. (Hollingsworth et al., 2009)

Two proposals for a plant barcode were submitted to the CBOL. The first was from Kew Royal Botanical Gardens, who came up with two options for combinations of regions to serve as barcodes. Option one included the plastid genes *rpoC1*, *rpoB* and *matK*. Option two included *rpoC1, matK*, and *trnH-psbA*. The reason for submitting two options was the uncertainty of whether or not *trnH-psbA* would fit into the protocols of the CBOL, due to its highly variable length (Kew, Royal Botanical Gardens 2009). The second proposal, from the CBOL plant working group was for the plastid genes *matK* and *rbcL*. One of their original proposals was to include *trnH-psbA,* but a decision was made against this to avoid the added cost and time of sequencing three loci, and the potential delay to finding a barcode by including *trnH-psbA* (Hollingsworth et al., 2009). The reason for these choices are summarised in Table 3, but basically none of the regions fulfil all of the criteria alone and a combination poses more chance of identification.

Table 3: A summary of analysis of candidate barcode regions against criteria

Name	Function	Positive attributes	Negative attributes
rpoC1	Coding plastid region	High quality sequence data produced. Routine PCR. Universal	Discrimination low.
rpoB	Coding plastid region	High quality sequence data produced. Routine PCR. Universal.	Discrimination low.
matK	Coding plastid region	Discrimination high.	Discordance between forward and reverse reads of sequencing. Requires manual editing of sequence traces.
trnH-psbA	Intergenic plastid spacer	Routine PCR. Discrimination high.	Poor quality sequence data due to high levels of mononucleotide repeats. Requires manual editing of sequence traces.
ITS	Intergenic nuclear rRNA spacer	High quality sequence data. Routine PCR	Not universal across all genera.
YCF5	Coding plastid region		Deleted from Bryophytes
accD	Coding plastid region		Missing from the grass family.
ndhJ	Coding plastid region		Truncated/non-functional in some Orchid groups.
psbK-psb1	Intergenic plastid spacer	Discrimination high.	Poor quality sequence data due to high levels of mononucleotide repeats.
atpf-atpH	Intergenic plastid spacer		Fell below accepted median for discrimination. Fell below accepted median for sequence data.
rbcL	Coding plastid region	High quality sequence data produced. Routine PCR.	Discrimination low.

(Adapted from Hollingsworth et al., 2009)

A committee was created of three independent reviewers who evaluated both proposals. The recommendation was for the three-locus proposal. The CBOL executive committee then evaluated the two proposals along with the recommendations made by the review committee. They decided that the three-locus proposal was more desirable, but the added cost and delay to finding a plant barcode swayed them to the two-locus proposal. In late 2009, the CBOL approved the two-locus proposal of the CBOL plant working group, with an 18 month review period (CBOL, 2012).

Since this period, much research has been occurring to develop plant barcodes. It has been demonstrated for some plants families that one barcode region is enough to allow discrimination between species. Pang et al 2011 have shown this with Rosaceae, Xiang et al 2011 with

Juglandaceae, and Song et al 2009 with Polygonaceae to name a few (Pang et al., 2011b, Xiang et al., 2011, Song et al., 2009). For some families two barcodes used together has been the answer; Hollingsworth et al 2009 used rbcL and matK for angiosperms (all flowering plant species) and Madesis et al 2012 for Leguminoceae (trnL and ITS2) (Hollingsworth et al., 2009, Madesis et al., 2012) . The two locus approach posed issues with alignment of highly variable regions such as those seen using trnH-psbA in some plant families. In order to overcome these issues, the 'tiered approach' was suggested (Newmaster et al., 2013). An easy to amplify region such as rbcL is used as a scaffold with which data from highly variable regions can be placed, such as ITS2. The favourite candidate for the so called second tier is ITS2 due to its high species discrimination abilities and short sequences. For herbal medicinal plants, ITS2 is able to overcome the degradation that often occurs from heavy processing. This approach can allow up to 80% of plant species to be identified, not an easy feat with some 400,000 species in existence (de Boer et al., 2015). A recent review by Parveen et al assesses the development of barcoding for herbal products and addresses the limitations. They state that barcoding of extract based products is not feasible which will be explored in this thesis. They also point out that due to the ubiquitous nature of DNA, that barcoding cannot be used to authenticate the presence of specific parts of plants. This has importance when the requirement for specific parts of a plant are needed for efficacy (Parveen et al., 2016).

1.4 *Actaea racemosa* L, Black Cohosh

Black Cohosh (*Actaea racemosa* L*.,* also known previously as *Cimicifuga racemosa* (L.) Nutt) is a perennial woodland plant native to North America and Canada, and is a member of the buttercup family (Ranunculaceae). The plant is tall with racemes of white flowers which blossom in the summer. The rhizome and the roots of the plant are used in phytomedicinal preparations used to treat menopausal-related complaints in Europe and as a dietary supplement for the same purpose in America. These remedies are available over the counter and as prescribed by practitioners (Denham et al., 2011). Above the soil the Black Cohosh plant is not unlike other *Actaea* species as can be seen

in Figure 1. Various American species also share the same habitats (Figure 2). The similar appearance and habitat makes contamination of harvested Black Cohosh highly possible (Mahady et al., 2008, Baker et al., 2012).

Actaea racemosa

Actaea podocarpa

Actaea heracleifolia

Actaea dahurica

Figure 1: A selection of *Actaea* plants native to Northern America and Asia – Similarity of appearance between species illustrated

(Images obtained from: (Missouri Botanical Gardens, 2017b, Mt Cuba Center, 2017, Longwood Gardens, 2017, Missouri Botanical Gardens, 2017a)

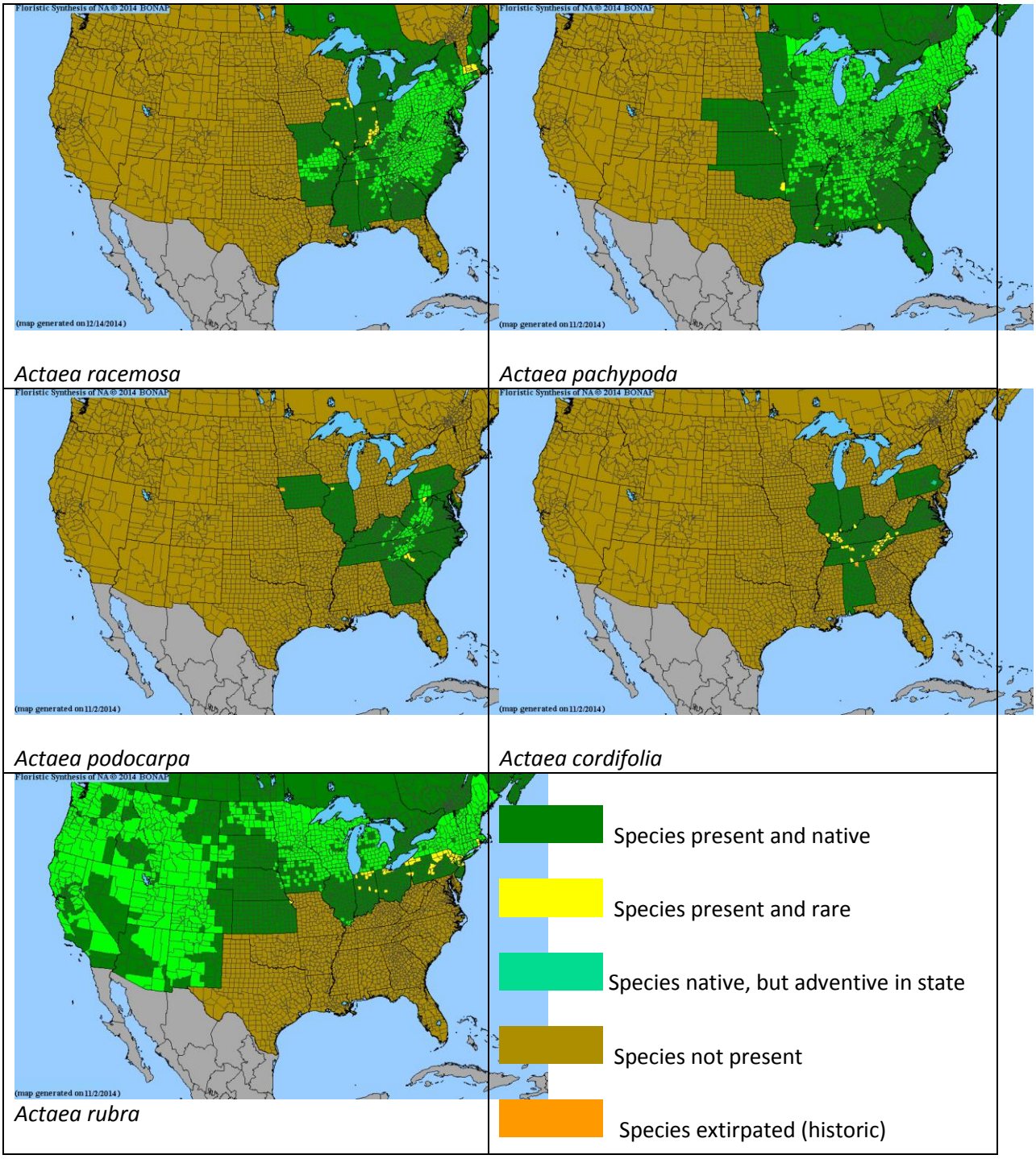


Figure 2: The presence of various *Actaea* species in Northern America showing overlap of habitat

[Available from: http://bonap.net/NAPA/TaxonMaps/Genus/State/Actaea] (Kartesz, 2015)

Historically, Black Cohosh has been used to treat a variety of ailments including anything from rheumatism to malaria (Masada-Atsumi et al., 2014, Foster, 2013), the Native Indians of American

have traditionally used Black Cohosh for a very long time (Betz et al., 2009) but today in the modern world it is used primarily for easing vasomotor symptoms e.g. hot flushes cause by the menopause.

1.4.1 Conservation of Black Cohosh

Black Cohosh is at risk of becoming an endangered species in some of its native wild habitats. Large scale commercial cultivation of Black Cohosh is almost non-existent; hence the plant is collected from the wild. Between 1997 and 2010, just under 33 tonnes of Black Cohosh raw material came from cultivation efforts, over 1,700 tonnes was sourced from wild harvesting (Gafner, 2016). Over-collection and threat to habitat are the primary threats to the wild population. The collection of the rhizome and roots leads to destruction of the plant, so collection causes a decline in numbers (Applequist, 2003).

The plants grow in Northern America and Canada. According to NatureServe (2010) areas of these regions where the plant has become endangered are; Massachusetts, Mississippi, and Illinois. The plant has become at risk in Ontario and Indiana. (NatureServe, 2010) (Figure 3).

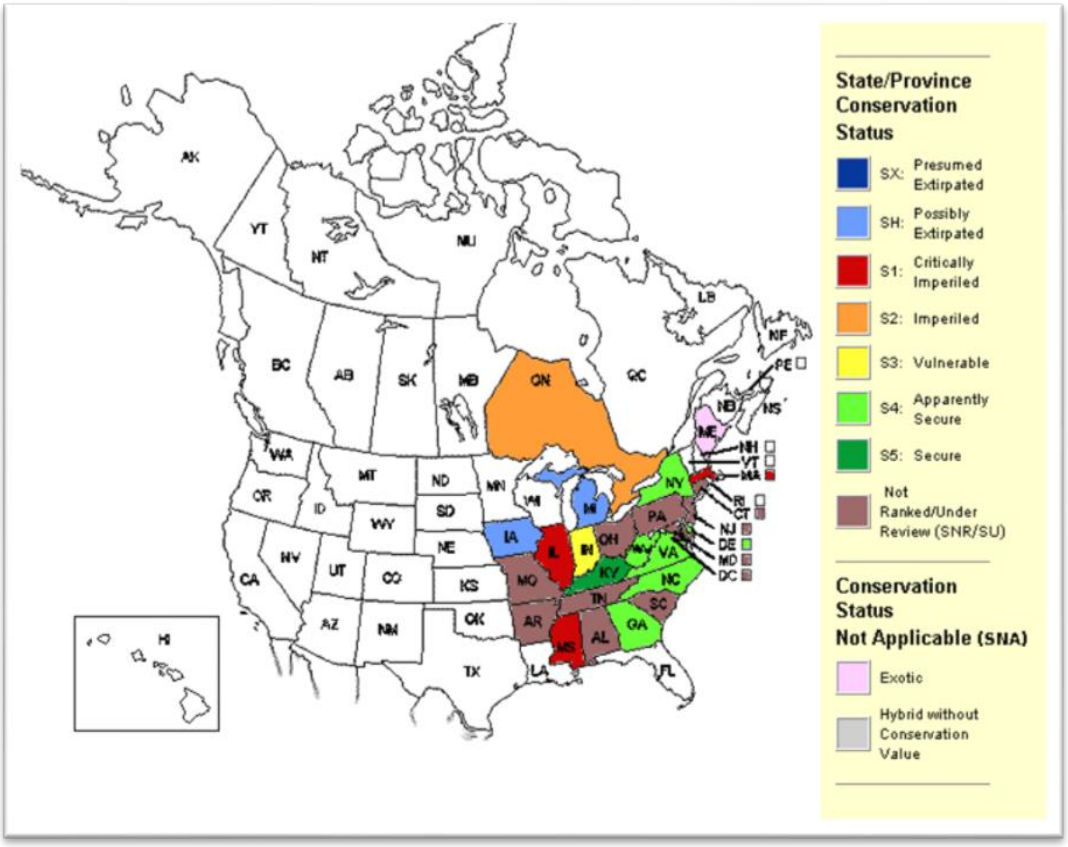


Figure 3: A map of Northern America and Canada showing Black Cohosh conservation status in different areas.

[Image available from: http://www.natureserve.org/explorer/]

CITES, the Convention on International Trade of Endangered species of Wild Fauna and Flora, is an agreement between governments across the globe. The purpose of CITES is to prevent the endangerment of species through international trade. Black Cohosh was considered for listing in Appendix II in 2001 and then Appendix III in 2002. The decision was made not to include the plant in either appendix as it did not fully meet the requirements of listing but its status was to be monitored. Although the species is endangered in some single American States, it is not classified as an endangered species due to its abundance in other States of America. *Actaea podocarpa* and *Actaea rubifolia* also share the same habitat and can be collected accidently. These species were also considered alongside *Actaea racemosa* for listing (USFWS, 2002). In 2005 the Medicinal Plant Working Group continued to collaborate with the U.S Forest Group and Garden Club of America to monitor the status of Black Cohosh. The idea was to ascertain the effect of varying levels of harvest.

Further collaboration with the IUCN-Medicinal Plant Specialist Group and the North American Pollinator Protection Campaign was established to produce fact sheets for native plants of the USA and included *Actaea racemosa.* The aim of these documents was to promote sustainable use. In the United States a permit is required to collect Black Cohosh from National Forest. Between 1995 and 1997 the number of permits purchased increased from 4 to 50 (Robbins, 1999). This is evidence of the increased demand for the plant.

The plant is listed as endangered on the Natural Resources Conservation Service website of the United States Department of Agriculture in Illinois and Massachusetts (USDA, 2017). This gives the plant a federal endangered status. It is not listed for Mississippi.

Conservation projects are up and running for Black Cohosh in parts of the US. In Massachusetts, Black Cohosh is covered by the Natural Heritage and Endangered Species Program and listed under the Massachusetts Endangered Species Act. This protects the plant against collection, killing, possession, sale and activities that could affect the mortality of the plant (NHESP, 2010). Figure 4 shows the extent of just how endangered *Actaea racemosa* has become in the State of Massachusetts.

Figure 4: A map of Massachusetts showing distribution of *Actaea racemosa*

Image available from: http://www.mass.gov/eea/docs/dfg/nhesp/species-and-conservation/nhfacts/actaea-racemosa.pdf (NHESP, 2010)

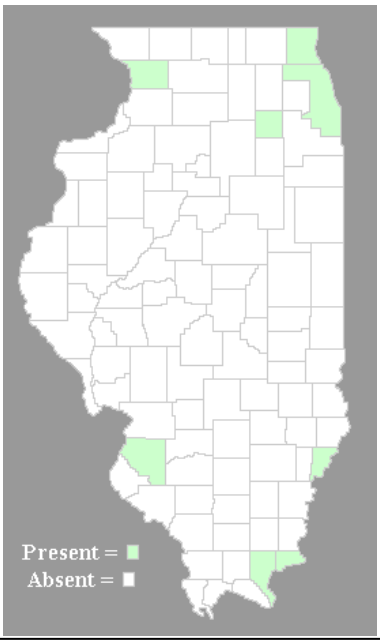


Figure 5: A map of Illinois showing distribution of *Actaea racemosa*

Image available from: http://www.illinoiswildflowers.info/woodland/plants/black_cohosh.htm

(Hilty, 2016)

Actaea racemosa is listed on the Illinois Endangered Species Protection Board's (IESPB) Checklist of Illinois Endangered and Threatened Animals and Plant list. It is worth noting that the plant is listed as *Cimicifuga racemosa* with a common name of false bugbane. *Cimicifuga rubifolia* (*Actaea cordifolia)* is listed as Black Cohosh on the official list. These names are not correct as Black Cohosh is officially the common name for *Actaea racemosa* and false bugbane refers to non *Actaea* species that are sometimes mistaken for true *Actaea.* This listing gives both plants protection through the Illinois Endangered Species Protection Act (IESPB, 2015).

United Plant Savers is a non-profit organisation in the US and through research and education they aim to conserve native plants. Black Cohosh is currently on their 'at risk' list and a recommendation has been made to abstain from wild harvesting at this time (UPS, 2013).

1.4.2 Efficacy of Black Cohosh

During the menopause oestrogen deficiency leads to vasomotor and psychological symptoms including; hot flushes, night sweats, mood and cognitive disturbances, sleep loss, vaginal dryness and loss of libido (Borrelli and Ernst, 2008). The usual treatment of these symptoms is hormone replacement therapy (HRT) but involves undesirable side effects (Pinkerton et al., 2009).

The first commercially available Black Cohosh product dates back to 1956, with Shaper and Brümmer releasing Remifemin, an isopropanolic *Actaea racemosa* extract based product. In the same period of time Kupperman provided a Menopausal Index, KMI which assigned a severity scoring based on symptoms. In 1976 Germany established The German Medicines Act, and this required efficacy of medicines to be proven. The German ministry of Health, in 1985, provided guidelines for conducting clinical trials. In 1986 the first randomised placebo controlled clinical trial was conducted using a Black Cohosh preparation. In 1994 Hauser released a Menopause Rating Scale (MRS) (Henneicke-von Zepelin, 2017). The first clinical trial concerned with dosage was conducted in 1995. The trial showed that safety was maintained up to 127 mg/day and efficacy was maintained as low as 40 mg/day. They also found that women in the perimenopausal stage were finding more relief from symptoms with 127 mg/day rather than 40 mg/day (Liske et al., 2002). 2003 saw the first clinical trial of an ethanol extract being conducted, Klimadynon from Bionorica. In the time between 2000 and 2015 there has been 28 clinical trials conducted all over the world. These trials have confirmed the efficacy of Black Cohosh extracts as a treatment for Menopause symptoms. In these trials Black Cohosh was superior to placebo and in some cases worked better than hormone replacement therapy. Other treatments areas have also been explored including osteoporosis fractures, breast cancer relapse, sleeping issues and cognitive abilities (Henneicke-von Zepelin, 2017).

Aside from clinical trials, there has been much scientific research into the efficacy of Black Cohosh. In 2005, Seidlova´-Wuttke found possible osteoporosis-preventing capabilities of Black Cohosh in aging males with reduced testosterone levels. Orchidectomized (testicles removed) rats were treated with

BNO 1055 and a statistically significant reduction in bone demineralization was observed (Seidlova-Wuttke et al., 2005). Another study in 2013 demonstrated that the German Black Cohosh product Remifemin® has the same protection against osteoporosis as hormone therapy in treated rats (Cui et al., 2013).

Hot flushes are the most prominent vasomotor symptom that menopausal women encounter. They are often accompanied by perspiration and heart palpitations. There does not seem to be a universal set of symptoms experienced by every woman, but factors such as ethnicity show evidence of influence, as 24% of Caucasian women experienced hot flushes compared to 37% of African-American women (Gold et al., 2006). Within the first few seconds of a hot flush episode, peripheral dilation, and increased skin temperature and blood flow occur.

In menopausal women, the reduction of oestrogen causes the pituitary gland to release high levels of luteinising hormone (LH) in pulses at regular intervals. These pulses are a result of activation of gonadotropin releasing hormone (GnRH) neurons in the hypothalamus, and subsequent release of GnRH into portal vessels connecting the pituitary gland to the hypothalamus. In ovariectomised (ovaries removed) rats, treatment with oestrogen showed that LH release was inhibited and therefore proves the effect of oestrogen on this process (Wuttke et al., 2014). The GnRH pulse generator becomes overactive due to erratic neurotransmitter release. Oestrogen regulates release of catecholamines, gamma aminobutyric acid (GABA) and serotonin in the hypothalamus which is important in episodes of luteinising hormone pulses. When oestrogen levels are lowered these compounds are released at much higher levels which come into contact with hypothalamic neurons responsible for thermal and cardiac regulation. Kapur (2010) demonstrated that treatment of *A. racemosa* extract BNO 1055 (a Black Cohosh extract manufactured by Bionorica) on ovariectomized rats reduced hot flushes. It did not affect uterine weight so is not oestrogenic (Kapur et al., 2010). Dopamine and serotonin inhibit pituitary release of LH. Black Cohosh has been shown by Powell (2008) and Burdette (2003) to contain serotoninergic compounds and Jarry et al 2003 showed

evidence of dopaminergic compounds being present (Powell et al., 2008, Burdette et al., 2003, Jarry et al., 2003, Wuttke et al., 2014).

Vaginal discomfort is another major issue related to the menopause. Vaginal pH is usually kept low by oestrogen, so becomes more alkaline during the menopause, leading to increased risk of infections (Pinkerton et al., 2009). In a clinical trial, Wuttke (2003) found that vaginal superficial cells significantly increased following treatment using BNO 1055, lowering vaginal pH and increasing vaginal lubrication (Wuttke et al., 2003).

1.4.3 Safety of Black Cohosh

The safety of use of Black Cohosh is a well-researched and documented area. Over the years there have been isolated cases reported of hepatotoxicity in connection with the use of Black Cohosh. There were also concerns for the effect of Black Cohosh on breast health due to the belief of the herb being oestrogenic.

In response to this, in 2004 a small workshop was held with participation from the National Centre for Complementary and Alternative Medicine (NCCAM), National Institutes of Health (NIH), the NIH Office of Dietary Supplements (ODS), the Office of Research on Women's Health, NIH: the National Cancer Institute, the National Institute on Aging, the US Food and Drug Administration, the American Herbal Products Association and the Centre for Science in the Public Interest. A further workshop was held in 2007 and a number of recommendations were made, mostly in regards to clear reporting of adverse events, improvements in communication and more thoroughly documented clinical trials. Also further research into safety, clinical benefit, mechanism of liver toxicity, adulteration, appropriate chemicals markers and mechanism of action studies to be carried out on Black Cohosh products (Betz et al., 2009)

A problematic area was the inconsistencies found with different conducted clinical trials. Several studies found that Black Cohosh reduced vasomotor symptoms to the same degree as hormone

replacement therapies and was superior to placebo (Nappi, 2005, Osmers et al., 2005, Wuttke et al., 2006, Molla et al., 2011). On the other hand another trial found that Black Cohosh had very little effect compared to hormone replacement therapies (Newton et al., 2006). In 2013 Beer and Neff reviewed available clinical trial information with an aim to determine if there was a difference in efficacy from registered products to non-registered (Beer and Neff, 2013). They found that registered products demonstrated efficacy in all clinical trials. The clinical trials which failed to prove the efficacy of Black Cohosh were found to have used an unregistered product. Interestingly the trial mentioned in the previous paragraph (Newton et al., 2006) was found to be an American dietary supplement rather than a registered natural medicine. Registered isopropanolic extract products have several clinical trials that are able to support efficacy in all cases. The types of trial range from randomised controlled, uncontrolled and controlled, and included thousands of women. Trials for ethanolic Black Cohosh extracts, although on a smaller scale and lesser in number compared to isopropanolic extract trials, are able to prove efficacy also. Combination products of Black Cohosh and St John's Wort were also trialled and evidenced efficacy in both treatment of vasomotor symptoms along with psychological complaints.

Table 4: A table to show the relationship between efficacies of products in trials to type of product

All clinical trials	Registered herbal medicinal product	
Evidenced efficacy?	Yes	No
Yes	15	1
No	0	2

Problems with the identification of *Actaea racemosa* by the use of questionable chemical marker compounds have been found. The marker compound outlined in the Ph. Eur is the triterpenoid glycoside, 23-Epi-26-deoxyactein for identification (EDQM, 2017a) with the use of thin layer chromatography. This compound was found to be present by Gafner (2006) in other species of *Actaea,* and so is not a good marker for *A. racemosa* (Gafner et al., 2006). A clear marker needs to be found and utilised, so confidence in the identification of *A. racemosa* is definite. He (2006) found

that *A. racemosa* can be distinguished using high performance liquid chromatography (HPLC) methodology with the marker cimiracemoside A, another triterpene glycoside, as it was found solely in *A. racemosa* amongst 10 *Actaea* species (He et al., 2006).

The current Ph. Eur monograph contains methods to discriminate between *A. racemosa* and four other closely related *Actaea* species.

The main issues discussed in the 2004 workshop were those related to safety (Betz et al., 2009). There were concerns about the use of Black Cohosh in individuals with breast and other cancers. This concern originally rose over speculation that Black Cohosh had oestrogen-like activity. This was a study where mice were treated with Black Cohosh extracts and breast cancer cells were also tested *in vitro* with Black Cohosh extracts. Further investigation showed that Black Cohosh did not in fact contain any compounds that could serve as ligands for oestrogen receptors (Liu et al., 2001). Rockwell (2005) tested 3 different Black Cohosh products on mouse breast cancer cells and found that none of the preparations affected the proliferation rate. They did however find that Black Cohosh increased the levels of cytotoxicity of cancer fighting drugs, docetaxel and doxorubicin and decreased the cytotoxicity of cisplatin (Rockwell et al., 2005). Other studies carried out since (Bolle et al., 2007, Hostanska et al., 2007) found no ill effect of Black Cohosh on breast tissue, so this deemed the drug safe for non-cancer patients but questionable for cancer patients receiving therapy. Beer and Neff (2013) also reviewed safety from 41 trials aimed at investigation of safety. The general safety trials showed that Black Cohosh gives no side effects as when compared to placebo, Black Cohosh did not differ significantly in results. Two investigations that were analysed showed no significant interaction with cytochrome P450 isoenzymes and P Glycoproteins, and therefore no drug interactions involving these pathways would be seen. Studies into effect of Black Cohosh on the breast and uterus were also examined. Of the studies examined 22 delved into safety of oestrogen sensitive organs. They found that in all cases there were no clinically significant changes in oestrodiol, follicle stimulating hormone (FSH) or luteinising hormone (LH). The following were also

unaffected; endometrium, breast density, breast cell proliferation or cell morphology of nipple aspirate fluid. Trials were also conducted into breast cancer risk. The previous suspicions that Black Cohosh increased breast cancer risk were shown to be unfounded, and the isopropanolic extracts were actually shown to decrease the risk of breast cancer (Beer and Neff, 2013). In 2007 a clinical trial showed that the use of Black Cohosh increased remission to 4.5 years when using an isopropanolic extract product (Henneicke-von Zepelin, 2017).

The next area of safety concerns the liver. For a number of years now, Black Cohosh has been suspected of causing hepatic damage. Many types of liver injury have been reported ranging from liver failure to autoimmune hepatitis, from patients who were taking a preparation containing Black Cohosh.

In 2002 an Australian report highlighted six cases of hepatitis following the use of herbal drugs. Two of these patients were taking Black Cohosh preparations (Whiting et al., 2002). Since the release of this report many other reports have been made. The reports have been reviewed extensively by various organisations. The European Medicines Agency reviewed 44 of the reports in 2008. They found only 18 of the reports to be documented adequately, three of these were considered to contain evidence of a possible relationship with Black Cohosh and two were considered to have a probable relationship with Black Cohosh. These five reports were then reviewed by a hepatologist who found no relationship between Black Cohosh and liver damage in four out of the five. The remaining report was deemed to be unlikely to be due to Black Cohosh (Teschke and Schwarzenboeck, 2009). Levitsky (2005) found that the patient of one of the cases judged to be of probable nature had been receiving 10 times the recommended dose. (Levitsky et al., 2005) Later in 2008, Levitsky published an erratum declaring that the report in question originally stated:

“The patient did not drink alcohol or use drugs and was not taking any medications including other herbal medications, acetaminophen, or nonsteroidal anti-inflammatory drugs.”

It was later found that the patient regularly consumed wine and had been taking drugs known to influence the health of the liver. (Levitsky et al., 2008)

The Medicines and Healthcare Products and Regulatory Agency of the UK received around 21 reports of liver damage associated with the use of Black Cohosh. All cases underwent detailed analysis which led to the decision that although adverse reaction was rare the following actions would be taken:

- Warnings to be added to the product information on licensed and unlicensed products regarding rare adverse reactions of the liver.
- Monitoring of further adverse reactions suspected to be linked to Black Cohosh
- Encouragement for licence holders of Black Cohosh products to carry out research into mechanisms of how Black Cohosh may cause liver damage. (MHRA, 2006)

Warning labels were also issued in Australia after three serious cases of liver damage were reported with one leading to death of the patient and all three receiving liver transplantation. There was not enough information available to link these cases to Black Cohosh but it was cause enough to issue very strong warning labels (Therapeutic Goods Administration, 2006, Therapeutic Goods Administration, 2007).

In 2008 the United States Pharmacopoeia (USP) analysed 30 non-duplicate reports associated with Black Cohosh and hepatotoxicity. After assigning possible causality to each of the cases, they found none to be certain or even probable. The problem with most of these reports was that other factors were present which could also be causing the effects. Some of the patients were reported to be consuming alcohol, taking other synthetic drugs known to affect the liver, taking unlicensed products and taking poly herbal remedies containing other herbs associated with hepatotoxicity such as skull cap, Scutellaria species (Lamiaceae). One patient 'recalled seeing the words black Cohosh on the bottle but could not remember any details about the label or the bottle which she

had discarded', so it could be possible that the patient was not taking Black Cohosh or taking an unlicensed product containing other species of plants. One patient was detailed as taking 1000 mg daily of black Cohosh extract alongside two glasses of wine and various medications including acetaminophen which is known to be toxic to the liver in excess and also tested positive for hepatitis B surface antibody and herpes simplex virus immunoglobulin M. Another patient was detailed with taking 500 mg of black Cohosh extract daily but regularly consumed wine, used ibuprofen and used valacyclovir which is known to influence liver enzyme levels.

In 2002 Black Cohosh was given a Class 1a rating;

"Class 1a: Articles for which the Committee is aware of limited human scientific data concerning safety of the article but is unaware of significant safety issues that would prohibit monograph development when the article is used and formulated appropriately"

The USP concluded that the link between cases of hepatotoxicity and Black Cohosh was weak due to following

- incomplete case information and unknown products
- Variables present such as alcohol consumption
- Other medications being taken
- Risk factors pre-existing.

Even with the decision that the link was weak, the Dietary Supplements Information Expert Committee (DSI EC) decided to reclassify Black Cohosh into Class 2. The reasons given were that the possible adverse reactions were of a serious nature and there were an ever increasing number of adverse reports being made.

"Class 2; Articles for which the Committee is unaware of significant safety issues that would prohibit monograph development when the article is used and formulated appropriately, provided there is a warning statement in the labelling section"

The DSI EC decided upon the following wording for a labelling statement;

"Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice." (Mahady et al., 2008)

Health Canada received four reports of liver damage connected with Black Cohosh use. All four of these cases were deemed as serious, three of them were found to have a possible link and one to have a probable link. A more recent search through the Health Canada Vigilance Adverse Reaction Online Database brings up 9 reports of adverse reactions associated with Black Cohosh use. Again provided information is limited, although 6 of the 9 reports give a brand name of product (Painter et al., 2010, Betz et al., 2009). In 2011, a number of cases from the aforementioned were studied again by Teschke et al. They scrutinised the lack of information that was available from the authors of the case reports and also gave differential diagnoses. This is shown in Table 5 which was adapted from the publication (Teschke et al., 2011).

Table 5: A summary of lack of information provided from 40 cases of reported Black Cohosh liver toxicity

Information presented	Availability from 16 individual case reports	Availability from 24 individual spontaneous reports
Brand name	6	17
Manufacturer	4	9
Plant part	6	3
Solvent	2	0
Daily dose	9	6
BC Drug	1	5
BC Polyherbal product	4	12
Date of BC start	4	11
Date of BC End	1	8
Date of symptoms	2	14
Temporal association	8	8
Time on BC	13	8

Tim to onset	14	9
ALT Value (Alanine amino transferase	16	5
ALP Value (Akaline phosphatase)	14	4
Hepatotoxicity criteria	15	4
ALT dechallenge	4	1
Biliary tract imaging	12	2
HAV (Hepatitis A virus)	16	2
HBV (Hepatitis B Virus)	15	2
HCV (Hepatitis C Virus)	16	2
CMV (Cytomegalovirus)	11	2
EBV (Epstein-Barr Virus)	11	2
HSV (Herpes Simplex Virus)	4	0
VZV (Varicella Zoster Virus)	1	0
Comedication/Herbal mixture	11	19
Undetermined BC Product	10	7

(Adapted from (Teschke et al., 2011)

According to Teschke et al 2011, there are certain published elevated liver enzyme parameters that must be seen in tests to be able to attribute a case to being a liver injury. In Table 5 the spontaneous cases are particularly lacking these criteria. A very small proportion of the cases (10%) document a re-exposure test and in all cases was negative. Challenge, de-challenge and re-exposure are all clinically relevant aspects of assigning causality and were rarely documented (Teschke et al., 2011). A number of cases where positive re-exposure was documented were reviewed in 2014. None of the cases linked to Black Cohosh were found to have a positive re-exposure (the same negative reaction originally seen when the patient was re-exposed to Black Cohosh) (Teschke et al., 2014).

In recent years there have been further case reports of hepatotoxicity associated with Black Cohosh use. In Table 6 these have been briefly summarised from what was available and are still lacking in the basic information required from the criteria described by Teshcke et al 2011. Some of these case reports even reference and agree with Teshcke et al 2011 yet still miss the required information to assign causality to Black Cohosh (Teschke et al., 2011).

Table 6: A summary of lack of information and causality in more recent cases reporting hepatotoxicity from Black Cohosh use

Source	Liver Injury	Product	Comments
Franco et al 2017	Abnormal liver enzymes	150 mg root extract/day for 1 week	Very short usage time to be the cause and brand not given
Muqueet-Adnan et al 2014	Liver cirrhosis	Unknown brand or dose for 1 month	The authors declared that they could not firmly link this case to Black Cohosh as they are unaware of the Brand and purity of the product the patient was using
Enbom et al 2014	Abnormal liver enzymes	Unknown	Products were unknown as was duration of usage
Lim et al 2013	Sub-acute liver failure	Unknown brand or dose for 2 weeks	As the brand was unknown it is not certain if a registered or authentic product was used

(Franco et al., 2017, Muqeet Adnan et al., 2014, Enbom et al., 2014, Lim et al., 2013)

Adulteration of Black Cohosh products may explain the cases of hepatotoxicity. Similar binomial and common names can cause confusion as outlined in Section 1.4.4: Classification of Black Cohosh. Similar ground appearance and habitat sharing of different *Actaea* species also can cause misidentification as outlined in the start of Section 1.4 *Actaea racemosa* L. Black Cohosh. In the previously mentioned Health Canada case reports, the Black Cohosh product from the probable case was identified and tested using high performance liquid chromatography-mass spectrometry fingerprinting. The product was found to contain no *A. racemosa* and was instead another undeclared *Actaea* species, this led to the recall of 7 products and the discovery of 3 more substituted products (Jordan et al., 2010).

Jiang et al 2006 tested 11 commercially available products from the American market. Of the 11 products 8 were found to contain *Actaea racemosa.* They stated that 4 of the products that were tested were found to be adulterated with Asian *Actaea* species. They also quantified the amount of triterpene glycosides present, as many of these products claim that standardisation of 2.5% triterpene glycosides is achieved. They found that this varied dramatically, although they didn't

make any assumptions and commented that different methods of standardisation could be used. They also performed stability testing on a chosen product to decide if age of the product would be a factor on chemical constituent differences. The product was tested over 30 months and the findings showed that no qualitative or quantitative changes occurred (Jiang et al., 2006, Jiang et al., 2011).

1.4.4 Classification of Black Cohosh

The scientific name and classification for black Cohosh has changed numerous times over the centuries, which no doubt has led to much confusion. In 1705, Plukenet first classified Black Cohosh as *Christopheriana facie, Herba spicata, ex Provencia floridiana*. Between 1705 and 1750 it was most commonly known as *Actaea* with some botanists using *Christopheriana*. The first Linnaean name was *Actaea*, but was later split to include *Cimicifuga* for the presence of dry follicles instead of the fleshy unicarpellate berries characteristic of *Actaea* species. Rafinesque renamed the plant as *Macrotrys* based upon the decision that it did not fit with Linnaeus' classification and Eton later adjusted the name to *Macrotys*. The name was reverted back to the Linnaean *Cimicifuga* genus until 1998, when the morphological characteristics and genetic sequences were thoroughly explored by Compton et al 1998, who reclassified Black Cohosh along with all other *Cimicifuga* and *Souliea* species to *Actaea*. After this work the genus contained 28 species, 8 from North America, 19 from Asia and one from Europe (Compton et al., 1998). Two further species have been accepted following this work, both are native to America.

Table 7: A list of all species included in the *Actaea* genus

Name	Origin
***Actaea arizonica* (S.Watson) J.Compton**	North America
***Actaea asiatica* H.Hara**	Asia
***Actaea austrokoreana* (H.W.Lee & C.W.Park) Cubey**	Asia
***Actaea bifida* (Nakai) J.Compton**	North America
***Actaea biternata* (Siebold & Zucc.) Prantl**	Asia
***Actaea brachycarpa* (P.K.Hsiao) J.Compton**	Asia
***Actaea cimicifuga* L.**	Asia
***Actaea cordifolia* DC.**	North America
***Actaea dahurica* (Turcz. ex Fisch. & C.A.Mey.) Franch.**	Asia

***Actaea elata* (Nutt.) Prantl**	North America
***Actaea europaea* (Schipcz.) J.Compton**	Europe
***Actaea frigida* (Royle) Prantl**	Asia
***Actaea heracleifolia* (Kom.) J.Compton**	Asia
***Actaea japonica* Thunb.**	Asia
***Actaea kashmiriana* (J.Compton & Hedd.) J.Compton**	Asia
***Actaea laciniata* (S.Watson) J.Compton**	North America
***Actaea × ludovicii* B.Boivin**	North America
***Actaea mairei* (H.Lév.) J.Compton**	Asia
***Actaea matsumurae* (Nakai) J.Compton & Hedd.**	Asia
***Actaea pachypoda* Elliott**	North America
***Actaea podocarpa* DC.**	North America
***Actaea purpurea* (P.K.Hsiao) J.Compton**	Asia
***Actaea racemosa* L.**	North America
***Actaea rubra* (Aiton) Willd.**	North America
***Actaea simplex* (DC.) Wormsk. ex Prantl**	Asia
***Actaea spicata* L.**	Asia
***Actaea taiwanensis* J.Compton, Hedd. & T.Y.Yang**	Asia
***Actaea vaginata* (Maxim.) J.Compton**	Asia
***Actaea yesoensis* (Nakai) J.Compton & Hedd.**	Asia
***Actaea yunnanensis* (P.K.Hsiao) J.Compton**	Asia

The common names for *Actaea* species can include the term 'Cohosh'. For example, Black Cohosh is the commonly known name for *Actaea racemosa*, White Cohosh for *Actaea pachypoda* and Yellow Cohosh for *Actaea podocarpa*. This also leads to confusion with Blue Cohosh which is in fact a completely different plant from another family; *Caulophyllum thalictroides* (L.) Michx (Berberidaceae). There are alkaloids (sparteine and N-methycystisine) and saponins (cauloside A, saponin PE, and cauloside C) present in Blue Cohosh that have been reported to lead to birth defects, heart failure of neonates, and uterine stimulating effects, so confusion with this species can have dangerous consequences (Datta et al., 2014). Confusion is also caused by websites and Chinese suppliers referring to the Asian species, *Actaea dahurica*, as Black Cohosh (Mahady et al., 2008, Satchithanandam et al., 2008). Other completely non related genera can also be marketed as Black Cohosh such as *Serratula* (Asteraceae) and *Vernoni*a (Asteraceae) (Gafner, 2015, Gafner, 2016). Vernonia is an adulterant of Chinese *Actaea* products and so can become an adulterant of Black Cohosh products if adulterated by Chinese *Actaea* species.

Table 8 is taken from the Kew Gardens Plant Naming Service and gives the accepted name for *Actaea racemosa.* Table 9 gives a summary of other names for *Actaea racemosa* which have been found in scientific documents. *Cimicifuga racemosa* features in the most papers as it was the previously accepted name for the herb for many years.

Table 8: The number of sources using correct naming for *Actaea racemosa* versus total number of sources as detailed in the Kew Gardens Medicinal Plant Naming Service

Naming	Category	Sources using correct naming	Total sources checked
Actaea racemosa L.	Accepted scientific name from taxonomic source	18	123

Table 9: A breakdown of naming used for Black Cohosh in all checked sources from the Kew Gardens Medicinal Plant Naming Service

Scientific names as used in medicinal plant references	Records referring to name
Actaea monogyna	1
Actaea racemosa L.	18
Cimicifuga racemosa	1
Cimicifuga racemosa (L.) Nutt.	80
Cimicifuga racemosa Nutt.	7
Cimicifuga racemosa Nuttal	1
Cimicifugia racemosa	1

Kew Plant Naming Service [accessed from: http://mpns.kew.org/mpns-portal/searchName]

Table 10: A list of different binominal/common names for *Actaea* species and other possible adulterants

Species Name	Previous Name (pre 1998)	Common Name(s)
***Actaea racemosa* (Nutt.) L**	*Cimicifuga racemosa*	Black Cohosh, Black bugbane, Black snakeroot, Fairy Candle, Rattleweed, Squat Root.
***Actaea rubra* (Ait.) Wil/d**	*Actaea erythrocarpa*	Red Baneberry, China berry, Dolls eye.
***Actaea pachypoda* (Elliot)**	N/A	*Actaea alba*, White Cohosh
***Actaea dahurica* (Turcz. ex Fisch & C.A. Mey) Franch**	*Cimicifuga dahurica*	Xin gan sheng ma
***Actaea podocarpa* DC**	*Cimicifuga americana*	Yellow Cohosh, Dolls eyes, White Baneberry.
***Actaea simplex* (DC.) Wormsk. ex Prantl**	*Cimicifuga simplex*	*Actaea cimicifuga*, Var. Simplex. Black bugbane, Ye sheng ma
***Actaea heracleifolia* (Kom.) J. Compton**	*Cimicifuga heracleifolia*	Da san ye sheng ma
Actaea cimicifuga	*Cimicifuga foetida*	Chinese Cohosh, Sheng Ma,
***Actaea cordifolia* DC**	*Cimicifuga rubifolia*	Appalachian Bugbane
***Caulophyllum thalictroides* (L.) Michx.**	N/A	Blue Cohosh
***Vernonia aspera* (Buch.-Ham.) H.Rob.**	N/A	Rough leaf iron weed, Hei Sheng Ma
***Serratula chinensis* S.Moore**	N/A	*Rhaponticum chinense*, *Centaurea missionis*, Chinese

		sawort root, Guang dong sheng ma
***Astilbe biternata* (Vent.) Britton (Saxifragaceae)**	N/A	Bitter tea

1.4.5 Published monographs for *Actaea* species

The British Pharmacopoeia (BP) as well as the European Pharmacopoeia have legal status in the UK. The document is managed by the British Pharmacopoeia Commission and sponsored by the Department of Health. The BP also contains herbal monographs. The Black Cohosh monograph is identical to the Ph. Eur and in fact is extracted and referenced directly (BPC, 2017a).

The United States Pharmacopeia Convention governs and aims to standardise many aspects of medicine from drugs to food supplements. They produce various regulatory texts including the USP-NF (United States Pharmacopoeia and National Formulary) and the Dietary Supplements Compendium. Black Cohosh products are regulated as a dietary supplement in the USA. The text outlines *Actaea racemosa* as being the one and only species that can be called Black Cohosh (USPC, 2015).

The Pharmacopoeia of the People's Republic of China (PPRC) is managed by the Chinese Pharmacopoeia Commission (CPC). Although the PPRC does not include a monograph for Black Cohosh it does contain a monograph for Sheng Ma. Sheng Ma is a Traditional Chinese Medicine that is comprised of *Actaea dahurica*, *Actaea cimicifuga* or *Actaea heracleifolia*. The preparation is also called Rhizoma Cimicifugae which is also the name used in the Ph. Eur for Black Cohosh. This can lead to confusion (CPC, 2015).

The Japanese Pharmacopoeia is managed by the Pharmaceutical and Medical Devices Agency (PMDA). The publication includes an entry for Cimicifugae Rhizoma and is comprised of *Actaea simplex*, *Actaea dahurica*, *Actaea heracleifolia* and *Actaea cimicifuga*. All four species can be used interchangeably and all four species are identified with the same simple TLC based test. This involves

comparison to an isoferulic acid standard. Again this can cause confusion due to the similar Latin name to the authentic Black Cohosh product made with *Actaea racemosa* (PMDA, 2017).

The World Health Organisation (WHO) has a monograph collection with Black Cohosh included in the second volume of the series titled; WHO Monographs on Selected Medicinal Plants. This monograph usefully points out the difference in definition of Rhizoma Cimicifugae between the different published Monographs of different countries including those mentioned in this section. This monograph also includes information about clinical trials and other relevant research findings (WHO, 2004).

Another extensive monograph available is published by Frostburg state University of Maryland, USA. The series of monographs is titled Appalachian Plant Monographs. This monograph is more of a full comprehensive review than a classic monograph (Pengelly, 2011).

1.5 Authentication of Herbal Medicines

Ensuring that plant material is the authentic species is of utmost importance in the production of herbal medicines. There are many control measures put in place to assure that good quality material of the correct species is used. The use of reputable suppliers and security through the chain of custody is essential. Some manufacturers have custody from start to end, i.e. they have control over the growing of the plants through to packaging the finished goods and in this case the likelihood of adulteration or substitution becomes slim. In other cases, and much more commonly, the plant materials are purchased from wild collectors or agents who act as 'middlemen' and in others the plant may be purchased from another supplier readymade in extract form. The latter increases the chance of a herbal medicine being produced with contaminated or worse case the wrong material (Booker et al., 2012).

The responsibility of the manufacturer is to scrutinise the material to ensure it is fit for the consumer. This includes ensuring the species is correct using identification methods from visual

examination to state of the art analytical chemistry. It also includes all the methods set out in the earlier section 1.2.1.1 Monographs, which include heavy metal determination, detection of unwanted minerals and general foreign body detection. There is also a responsibility at all stages, for example storage and processing to maintain quality. The monographs are the minimum requirements for analysis of herbal material, most reputable manufacturers will have further in house quality control methods (Shinde et al., 2009).

As mentioned earlier, Black Cohosh has been found to be adulterated with Asian *Actaea* species, other American species that share the same habitat and different families that can appear similar such as *Astilbe* or *Caulophyllum*. This has been attributed to the fact that Black Cohosh is predominantly harvested from the wild, restricted for collection in some areas due to conservation and demand for the herb has increased over the years. This has been termed as 'economical motivated adulteration (EMA)' (Cumberford, 2012). Adulteration of plant material is more difficult to recognise than substitution and this is where the importance of research into identification methods is a crucial area for the integrity of finished products. Adulteration is defined as the accidental or deliberate addition of another substance to an item to increase the quantity. Substitution is defined as the replacement of the intended material with other parts of the same plant, different species from the same family or from other families. Substitution can be acceptable if the substitute material has the same pharmacological effect. In the case of this work substitution is discussed as a replacement of the intended material for inferior material.

Quality assurance covers a wide range of matters that influence the quality of products. Quality assurance includes the development stage, quality control testing, production, distribution and auditing. Quality control is the inspection of products to determine if defined characteristics are being met as an output of a process. This can involve measuring, examining or testing. Assurance of quality in the production of a herbal medicine can begin as early as seed procurement for cultivated material and the harvesting stage when collection occurs from the wild. Good practice involves

gaining as much information as possible including the name of the collector, date of collection, exact site of collection, which part of the plant was collected, organoleptic assessment where applicable, how the material has been processed and photographs or sketches of the plant (above ground plant and roots if necessary). A sample of the plant should also be collected and retained for reference. Ideally the reference sample should be incorporated into a herbarium and should be annotated with features of the plant used for identification (Smillie and Khan, 2010).

Since the first use of medicinal plants dates back thousands of years, the original method of identification was morphological examination due to available equipment and expertise. This is still a valid technique in modern times but has become 'unfashionable' with the introduction of chemical techniques, and has led to a decline of interest in the area. Although chemical techniques are well established, the major flaws of the methods remain a problem. Products can be 'spiked' with the chemical markers used for identification and the chemical profile of a product can be altered by several factors including genetics, age and environmental (Yuk et al., 2016). This is where the proposal of using Deoxyribose nucleic acid (DNA) becomes attractive. Techniques are available that can identify and quantify a sample simultaneously. These eliminate the problems with chemical techniques as DNA is not so affected by factors such as age or environment. With the efforts of the Barcoding of Life initiative, the use of DNA techniques in the identification of plants is becoming more accepted as a valuable technique that can be used in unison with available chemical methods to overcome the flaws of each technique.

1.5.1 Morphology

Morphological examination includes macroscopic and microscopic technique. Macroscopic identification involves comparison by eye, of visual traits of whole or large parts of a plant. Traits including, shape, size, colour, texture, taste and odour of flowers, fruits, stalks and leaves are compared to a reference sample. Microscopic identification entails inspection of powdered samples,

this includes inspection of cellular shape and size, colour under different solutions and presence of characteristic plant structures (Smillie and Khan, 2010).

1.5.1.1 Morphological Identification of Black Cohosh

Macroscopic identification of *Actaea racemosa* is possible by a very skilled and experienced taxonomist. Microscopic identification is also possible. There are variances present in the roots which can help distinguish *A. racemosa* from other related species. Figure 6 shows an illustration by Curtis Gates Lloyd and John Uri Lloyd. This shows clear distinctions in the root anatomy of *A. racemosa* and *A. podocarpa*. These observations were later backed up by photomicrographs produced in 1943 By Youngten. The work of Applequist (2003) further supports that *A. racemosa* can be distinguished from other *Actaea* species if whole roots are available to examine (Applequist, 2003). This is unlikely to be the case with most samples as they will have often been powdered or shredded. The Monograph supplied by the European Pharmacopoeia states how to identify a powdered sample microscopically but this is fairly complicated and would require a skilled professional.

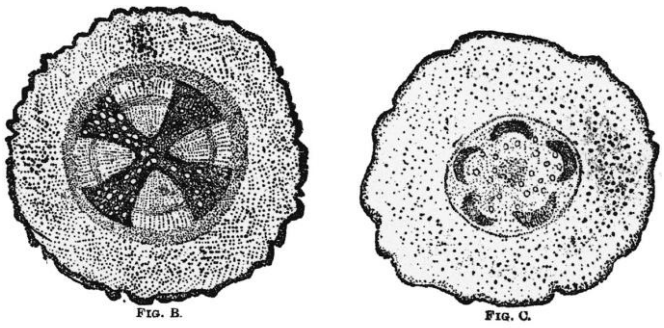


Figure 6: Sketches by Lloyd & Lloyd (1884). Transverse sections of *Actaea* roots.

B = *A. racemosa*. C = *A. podocarpa*.

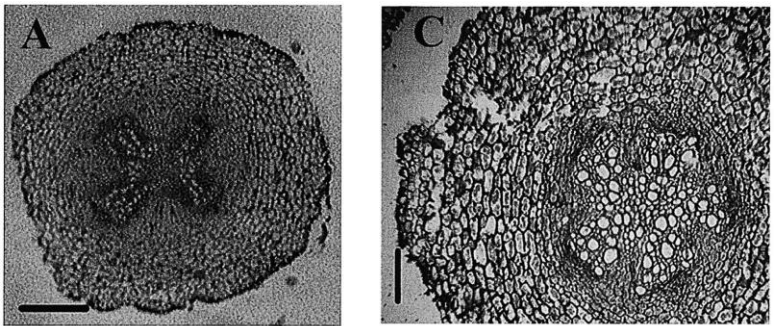


Figure 7: Photomicrographs of *Actaea* root sections produced by Applequist (2003).

A = *A. racemosa.* (Scale=500µM) C = *A. podocarpa*. (Scale= 200µM)

The Ph. Eur states that upon macroscopic observation, the rhizome is dark brown, knotted, hard and subcylindrical. The exterior shows branches terminating in the remains of a bud or cup shaped scar. The size ranges from 1.5 – 2.5 cm in diameter and can be anywhere between 2-15cm long. Transversely it presents a thin outer bark, this surrounds a ring of alternating pale vascular tissue and dark medullary rays, with a large central pith. The roots are dark brown, wrinkled, cylindrical/ obtusely quadrangular and 1-3mm in diameter. Transversely the outer bark is thick, containing a dark brown cylinder which houses a central region of 3-6 light coloured wedges of vascular tissue joint at the centre and separated by medullary rays. (EDQM, 2017a)

1.5.2 Chemical methods

Chemical identification tests are based upon finding chemical markers or a chemical profile unique to a particular species. There are many different methods involved but the most common methods involve chromatography and particularly include TLC and HPLC.

1.5.2.1 Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC)

TLC was developed in Russia in 1938 by Izmailov and was later made popular by Egon Stahl. Thin layers of absorbent silica gel are coated on to glass, metal or plastic. This makes up the stationary phase. A solvent is used as a mobile phase. The compounds are separated by movement of the

mobile phase over the stationary phase. It is simple, cheap, quick and easy to carry out. It does however have its disadvantages which include lack of full automation and reproducibility problems (Marston and Hostettmann, 2009).

HPTLC is an advanced development of TLC by utilising cutting edge technologies and instrumentation for refinement of all stages in the method. Equipment with the ability for precise plate preparation, controlled and reproducible plate development and analysis using state of the art software has allowed progression of the technique. HPTLC is also semi-automated only requiring transfer from one piece of equipment to another. This allows even junior scientists to perform the method with professional results. In basic terms the occurrence of human error can be eradicated in key stages as differences in or poor technique varied the results of TLC. Methodologies are constantly being developed which allows the use of standardised validated techniques. The particle size of HPTLC plates is much smaller than a TLC plate, which allows a shorter elution distance saving time and plate size. Vital factors such as humidity can be controlled allowing results to be reproducible from lab to lab. Samples are sprayed onto the plate in very small quantities reducing the volume needed and ensuring crisp bands are achieved further improving resolution (CAMAG, 2017).

1.5.2.1.1 Use of TLC and HPTLC for the identification of Black Cohosh

TLC is the method of choice for identification of *A. racemosa* and also the method of choice for detection of adulterant species, namely *C. foetida (A. cimicifuga*), *C. americana* (*A. podocarpa*)*, C. heracleifolia* (*A. heracleifolia*) and *C. dahurica* (*A. dahurica*). The main problem is that the TLC assay for *Actaea racemosa* is based upon detection of the chemical marker 23-epi-26 deoxyactein. As outlined in section 1.5.2.2, this compound is also found in other species of *Actaea.* The other potential issue is that this chemical compound can be added to products to pass for the desired product.

There is another method developed by CAMAG, combining HPTLC methods for triterpenes and plant acids. The method produces different banding patterns for several species but is limited to those species being significantly dissimilar enough for differentiation (Reich et al., 2008, Ankli et al., 2008).

This method has been further developed by Verbitsky et al 2008 by coupling TLC with bioluminescence. The preliminary work showed that *A. racemosa* could be distinguished from *A. podocarpa* and *A. pachypoda* (Verbitski et al., 2008).

Gaffner 2015 reviewed available (HPTLC) methods and found that they are similar. He also commented that the methods are proficient at identifying Black Cohosh in routine testing but, these techniques are not yet sufficient to detect adulteration. Figure 8 demonstrates the results of using HPTLC and also illustrates the ability of the method to differentiate between *Actaea racemosa* and American and Asian *Actaea* species by the different chemical profiles displayed (Gafner, 2015).

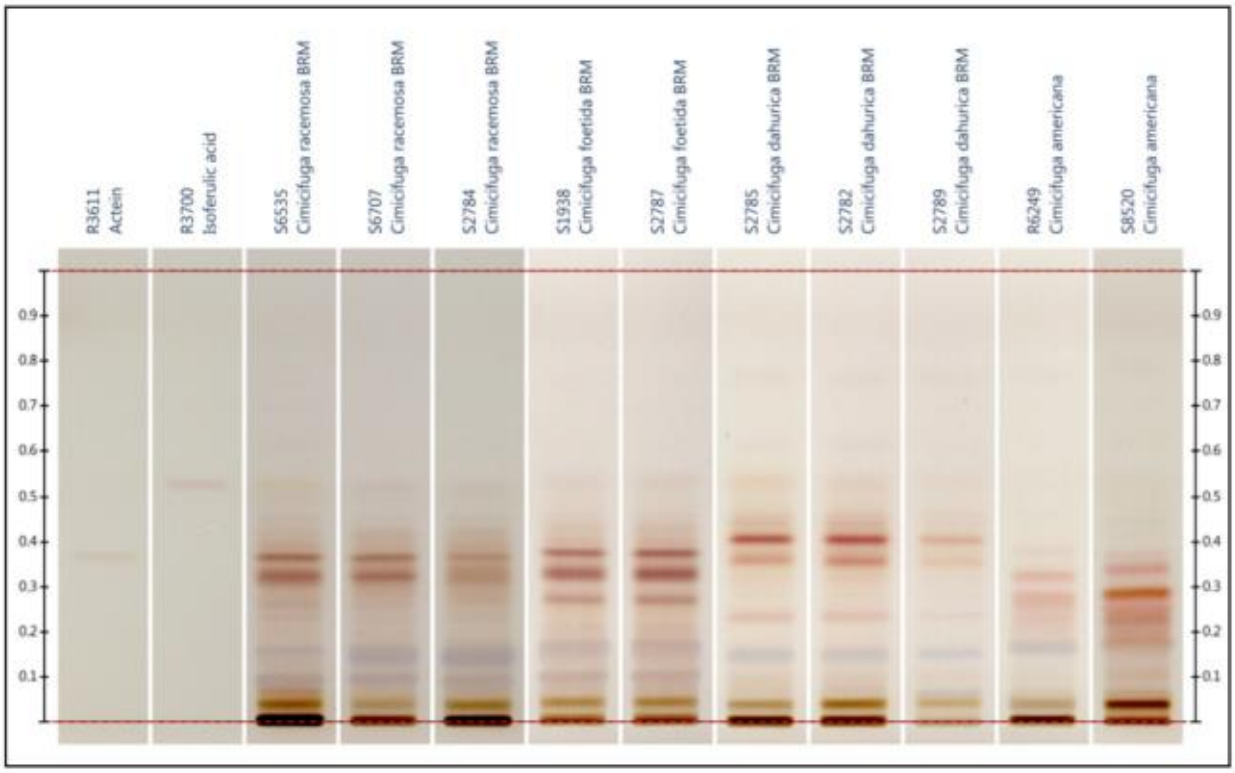


Figure 8: The A finished silica plate from a HPTLC experiment visualised with white light following derivitisation with sulphuric acid. Samples include *Actaea racemosa* and three other potential adulterant species and highlights differences in chemical profiles between the different species.

1.5.2.2 High Performance Liquid Chromatography (HPLC)

As long as there is a sample can be dissolved in a liquid, the ability to separate, identify and quantify its constituent compounds is possible by HPLC. The method is automated. The stationary phase of HPLC is within a coated column and the mobile phase is the solvent which is pumped through the column at high pressure (Marston and Hostettmann, 2009).

1.5.2.2.1 Use of HPLC in identification of *Actaea racemosa*

High performance chromatography-evaporative light scattering detection (HPLC-ELSD) method has been successful in discriminating *A. racemosa* from other *Actaea* species (He et al., 2006).

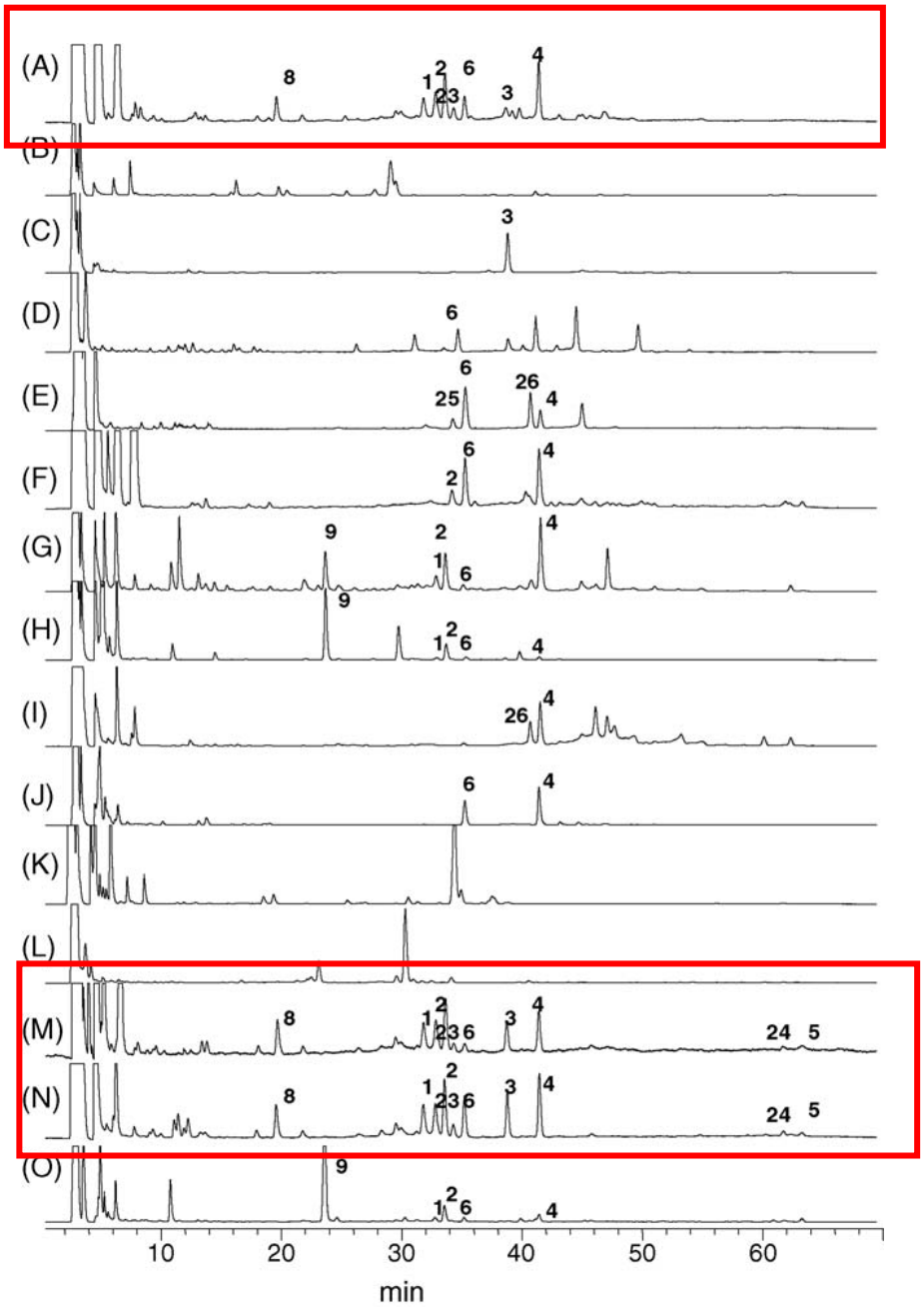


Figure 9: Fingerprint chromatograms obtained by Kan He et al 2006 by the HPLC-ELSD method

(A*) A. racemosa*, (B*) A. podocarpa*, (C) *A. cordifolia* (D) *A. acerina* (E) *A. biternat*, (F) *A. dahurica* (G) *A. cimicifuga* (H) *A. cimicifuga-2* (I) *A. heracleifolia* (J) *A. japonica* (K) *A. simplex* (L) *A. simplex-2* (M) Remifemim (N) Cimipure (O) Product B.

Compound 1 is 23-Epi-26-deoxyactein, which is present in *A. racemosa* and *A. cimicifuga.* This highlights that this is not an ideal marker for *A. racemosa* as it is present in other species. In other methods carried out in the same research paper, *A. dahurica* is also found to contain 23-Epi-26-deoxyactein. The only chemical constituent that is unique to *A. racemosa* is cimiracemoside A (peak

number 8 on Figure 9), which further research may prove to be a suitable chemical marker (He et al., 2006).

HPLC-ELSD has been further explored by Avula et al. The method that was previously developed by He et al 2006 was re-evaluated and streamlined (Avula et al., 2009).

More recently Geng et al 2017 developed a method which could differentiate *A. racemosa* from *A. dahurica, A. cimicifuga* (written as *A. foetida* in the article – confusion with the synonym *C. foetida*), *A. pachypoda* and *A. podocarpa*. This was an LC-MS method combined with principle component analysis. Feruloyl dopamine-*O*-hexosides were used as markers and patterns of markers were used to identify the species. This is demonstrated in Figure 10. It is worth noting that it was not possible to differentiate *A. dahurica* and *A. cimicifuga* (written as *A. foetida* in the figure) using this technique (Geng et al., 2017).

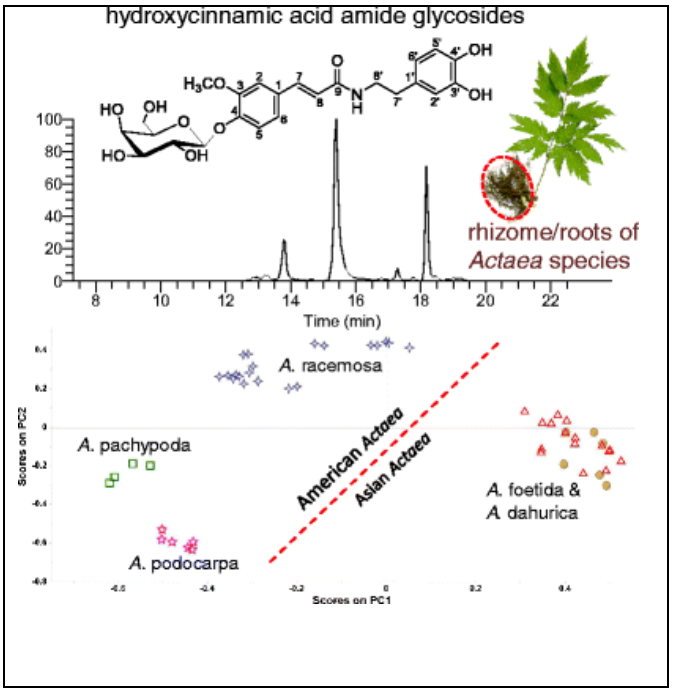


Figure 10: Principal component analysis of *Actaea racemosa* and 4 related species (Geng et al., 2017)

1.5.2.3 Other chemistry based methods

1.5.2.3.1 Mass spectrometry

Huang et al 2015 used mass spectrometry to develop a method capable of distinguishing three *Actaea* species including *A. racemosa, A. dahurica* and *A. podocarpa*. This was based upon chemical fingerprinting. The data was analysed using principal component analysis (PCA) which was able to differentiate the species. Although the authors state that the method is simple and quick, there is limitation to the number of differentiated species and the capital cost of purchasing such equipment is high (Huang et al., 2015).

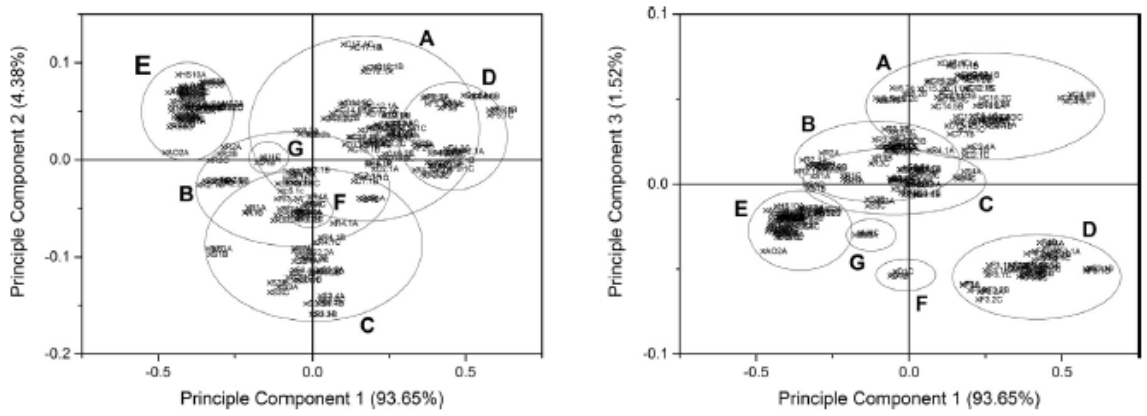
1.5.2.3.2 Nuclear Magnetic Resonance

Harnly et al 2015 conducted a combined study of mass spectrometry, NMR and DNA barcoding for Black Cohosh. The mass spectrometry section of the article is the same as what was described by Huang et al 2015 with the addition of further *Actaea* species; *A. cimicifuga, A. pachypoda* and *A. rubra.* Due to the complexity of the raw data the following species were analysed further; *A. racemosa, A. podocarpa, A. pachypoda and A. rubra.* An NMR based method was developed and compared to the mass spectrometry based method. In both cases the results were analysed using principal component analysis to cluster species together. In most samples identification matched for all three techniques. They did however find that a few of the samples did not fit in the respective clusters but were identified through DNA techniques. This shows how chemical methods can sometimes be wrong due to chemical constituent patterning in samples. NMR is the most costly out of the three methods from a capital perspective (Huang et al., 2015, Harnly et al., 2016).

1.5.2.3.3 UV Spectroscopy

Bittner 2016 used UV spectroscopy coupled with principal component analysis to attempt to differentiate *Actaea racemosa* from four other species; *Actaea simplex, Actaea heracleifolia, Actaea cordifolia* and *Actaea cimicifuga.* From the principal component analysis results it appears that it may be difficult to differentiate between *Actaea racemosa* and other *Actaea* species, as they cluster closely together as shown in Figure 11. The next step was to couple the UV method with LDA (Linear

discrimination analysis). This was able to differentiate the species more clearly as shown in Figure 12 for five of the included species (Bittner et al., 2016).



A- *A. racemosa*, B – *A. cordifolia*, C – *A. simplex* D – *A. cimicifuga*, E – *A. heracleifolia*, F – *A. dahurica*, G – Unknown *Actaea* species

Figure 11: Principle component analysis of 7 *Actaea* species, showing extensive overlap.

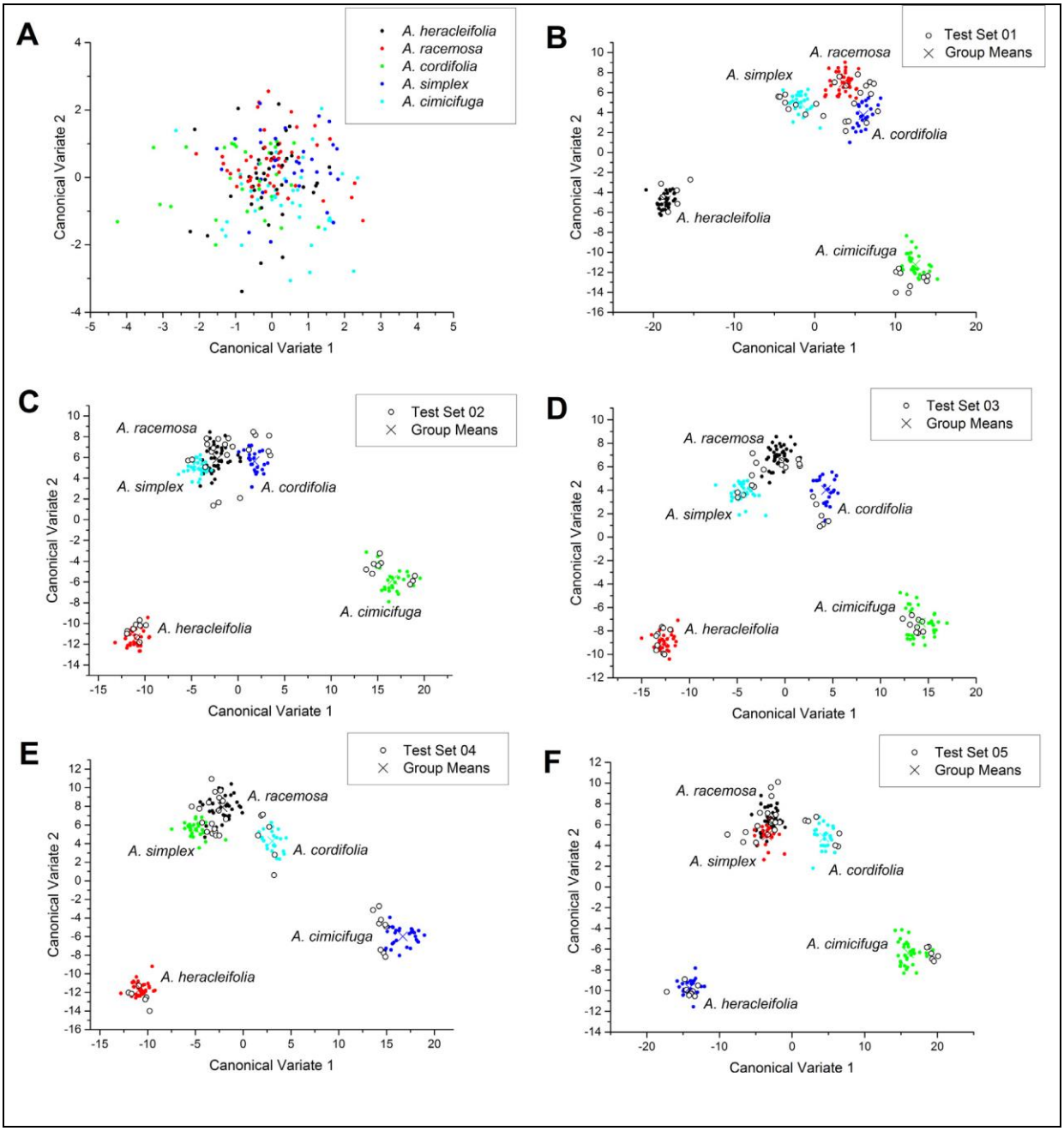


Figure 12: Linear discriminant analysis of *Actaea* species showing differentiation.

1.5.3 DNA based methods

The polymerase chain reaction (PCR) has revolutionised modern molecular science. The method allows exponential amplification of a particular DNA target in a cocktail of deoxynucleotide triphosphates (dNTPs), DNA polymerase and specific oligonucleotide pairs. The method is utilised in diverse regions of science from medicinal areas to agricultural applications.

There are many different DNA based identification methods in development by researchers across the globe. The biggest challenge is making these methods reproducible and easy to perform.

An example is restriction fragment length polymorphism (RFLP). This method is possible using restriction enzymes, which cleave DNA at particular sequences (restriction sites). After treatment with restriction enzymes, a DNA sample may be cut into different fragments depending on the presence of restriction sites. The digested DNA is analyzed using gel electrophoresis and the pattern of fragments obtained is indicative of what DNA is present. This technique is extremely specific but can take time and expertise to perform. Masuda 2016 developed an RFLP assay for the detection of *Actaea racemosa*. This was based on *Actaea racemosa* uniquely having the restriction site B*st*BI within the nrITS region, which resulted in two fragments on digestion with the restriction enzyme. This method was useful for detecting *Actaea racemosa* in products but as undigested fragments remained it was not reliable for detecting contamination. The next step was to develop an amplified refractory mutation system (ARMS) technique. The region *trnL-F* was utilised in this case and was found to more clearly identify *Actaea racemosa* from other closely related species. Eight Black Cohosh products from the Japanese market were analysed using this method and three of the products did not contain *Actaea racemosa.* To further validate the results TLC and HPLC analysis was completed which matched the ARMS results in terms of detecting corresponding marker compounds (Masada, 2016).

Another similar method is amplified fragment length polymorphisms (AFLP). This is based on the principles of RFLP. According to Vos (1995) who developed the method, there are three main steps. The first is digestion of the DNA using restriction enzymes and ligation of oligonucleotide adapters, the second is amplification of selected sets of amplified restriction fragments, and the third is gel electrophoresis of amplified fragments (Vos et al., 1995). Once established this method is very quick and simple to carry out, but the development is very challenging technically. This method has been

shown to be a tool in identification of *A. racemosa* and three adulterants, *A. pachypoda*, *A. cordifolia* and *A. podocarpa* (Zerega et al., 2002).

Random amplified polymorphic DNA (RAPD) is a technique that involves the use of sets of random primers. These primers anneal at various locations across the DNA sample and may or may not cause a region to be amplified depending upon the distance between them. Different banding patterns by gel electrophoresis will be the outcome and is reflective of which primer binding sites are present in the genome. It is a cheap and easy method to use but variance in results between labs occurs (Yip et al., 2007). RAPD analysis has been shown to be useful in identification of *A. racemosa* and adulterant species. Xu et al 2002 was able to discriminate *A. racemosa, A. podocarpa* and *A. cordifolia* respectively based on the production of different banding patterns (Xu et al., 2002).

Sequence characterized amplified region (SCAR) is an improvement of RAPD analysis. RAPD analysis is carried out and then identified polymorphic bands are excised from the gel and sequenced. Seethapathy et al 2014 used this technique to authenticate Ayurvedic medicines *Aconitum heterophyllum* and *Cyperus rotundus* respectively (Seethapathy et al., 2014).

STRs (short tandem repeats) or SSRs (simple sequence repeats) are repeated units of short nucleotide sequences, typically 2-6 base pairs long. They appear randomly in the genome of humans, animals and plants, and are the basis of the forensic method of RFLP. The number of repeats varies amongst individuals and the pattern of repeats across several loci can be used as an identification tool. Although the technique is established for use as a forensic tool in identification of human individuals, there is also much research being conducted to include identification of plants. The applications for this include identification and geographical placement of illegal drugs such as *Cannabis sativa* L. (Gilmore et al., 2003), food adulteration studies (Zhang et al., 2011) and several more examples. Yuan et al 2015 used this technique to build a cDNA library for the Chinese medicinal *Scutellaria baicalensis* with an aim to study evolution and genetic diversity (Yuan et al., 2015).

The most thorough and reliable identification technique is direct DNA sequencing (Sanger Sequencing). However, this can be expensive and time consuming, and difficult to apply to mixed, degraded or contaminated samples, so not always practical.

In the duration of this thesis there have been many advances in the barcoding effort for *Actaea racemosa.* Baker et al 2012 used the DNA barcode region *matK* to identify *Actaea racemosa* from other closely related species using Sanger Sequencing. The project involved four barcode regions also including nrITS2, *trnH-psbA* and *rbcL*, using 61 individual reference samples and 40 commercial product test samples. The success rate of the reference samples was good but the success rate of the commercial samples was lower. This is not surprising as the rigorous processing steps in producing herbal extracts can degrade the DNA somewhat. In these cases a barcoding primer pair targeted at an area with a smaller product outcome, *mini-matK*, was more successful. The purpose of mini barcodes is displayed in Figure 13.

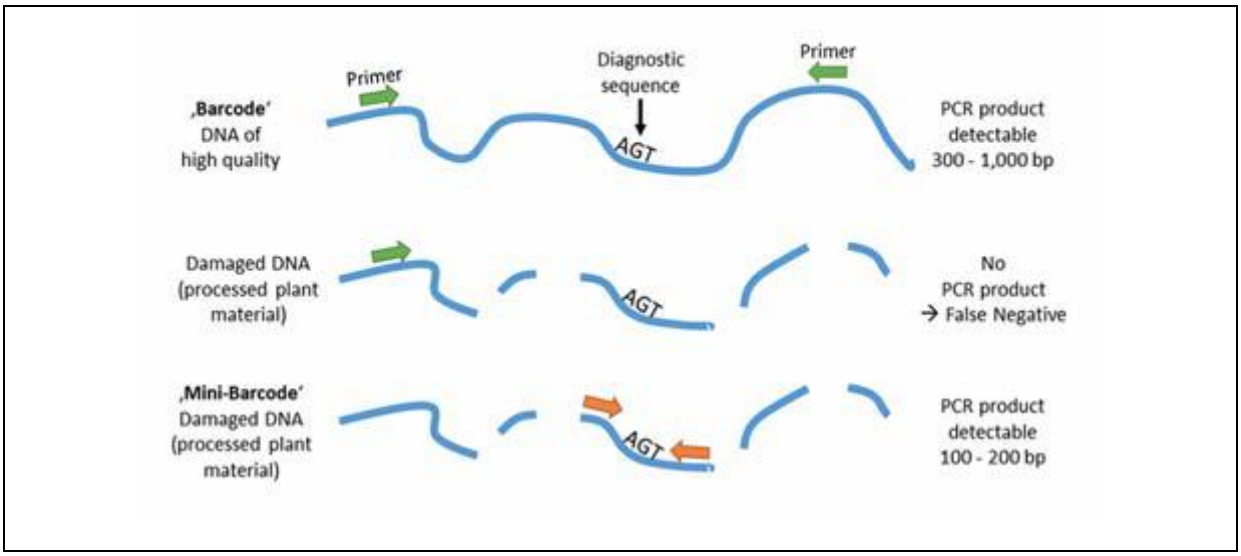


Figure 13: A diagram to show how mini barcode primers can overcome DNA degradation, allowing PCR amplification and thus enable sequencing (Parveen et al., 2016).

To validate their DNA method, the group obtained 31 vouchered *Actaea* samples from the New York botanical garden.

Table 11: A summary of the success rate of PCR amplification of four barcode regions in *Actaea* samples

Barcode region	Reference samples success rate	Commercial test samples success rate
matK	79%	0%
trnH-psbA	97%	10%
rbcL	100%	10%
nrITS2	100%	38%
Mini matK	Not used	90%

The sequence data was analysed to find differences in the barcode regions and allow identification of the individual species. Of 14 species they were able to find unique sequences in 7 species for nrITS2 and *tnrH-psbA*. Overall it was not possible to use one consistent barcode region for all 14 species included in the study, but they did show that the matK region can be used for identification of *Actaea racemosa* as it had a unique sequence to the other *Actaea* species. (Baker et al., 2012).

Masada-Atsumi et al 2014 conducted a study of 19 imported American Black Cohosh products, 6 non prescription Black Cohosh drugs from the European market and 5 samples of crude material available on the Japanese market used for production of Cimicifuga Rhizoma. The Japanese pharmacopoeia states four *Actaea* species for Cimicifuga Rhizoma; *Actaea dahurica, Actaea heracleifolia, Actaea cimicifuga* and *Actaea simplex.* DNA was extracted, the nrITS region was amplified and then sequenced using Sanger sequencing. Samples containing raw plant material were analysed with DNA techniques and the extracts were analysed using LC MS. Out if the 19 American products the group were able to sequence the nrITS region of 9. The region *trnH-psbA* was additionally sequenced for one of the products. They found that half of the products contained undeclared *Actaea dahurica.* The remaining products were dry extracts and could not be sequenced due to poor quality of DNA. All of the crude drug samples from Japan were found to be *Actaea dahurica* with one sample additionally containing *Actaea heracleifolia.* This is within the species prescribed in the Japanese Pharmacopoeia so legally acceptable to use in Japan for Sheng Ma but not for Black Cohosh. The samples were also analysed using LC MS. The European non prescription

drugs were all found to contain two commonly used marker compounds of *Actaea racemosa,* Actein and 23-*epi*-26-deoxyactein, but were not assigned a species due the inability to obtain DNA sequences. The other American products were found to contain 23-*epi*-26-deoxyactein. Some of the products contained Actein but some of the others contained slightly different compounds denoted as X and Y which were very similar to Actein. Two of the products contained compound Y. This was suggested to be acetylacteol 3-O-arabinoside, a compound unique to *Actaea cimicifuga*. There was a discussion of the suitability of Actein and 23-*epi*-26-deoxyactein as marker compounds for authenticating *Actaea racemosa* as it was detected in all samples. More work is needed for conclusive results for all the samples (Masada-Atsumi et al., 2014).

1.5.3.1 New DNA technologies

1.5.3.1.1 Next Generation Sequencing (NGS)

The downsides of Sanger sequencing can be overcome by next generation sequencing. A good example of the price difference would be that the human genome project cost £300 million when it was achieved. Today using Sanger sequencing this would cost £6 million. Next generation sequencing could achieve the same goal for £6000. It would also be much faster as instead of one single region up to 1kb being sequenced in each reaction, it can be done in parallel with many regions (Illumina, 2017).

Next generation sequencing (NGS) is a blanket term used to cover several different technologies which includes but is not limited to:

- Illumina sequencing (Illumina, 2017)
- Ion torrent: Proton/PGM sequencing (ThermoFisher, 2017)
- Roche 454 Sequencing (Roche, 2017)

The general principle of NGS is that large numbers of short reads are sequenced simultaneously and together build up a stronger result than classic sequencing with one sequenced strand. It is cheaper, more rapid and more reliable than Sanger Sequencing.

In short Illumina sequencing is based on the following steps; the template is enzymatically cleaved up (100-150bp), generic adapters are ligated to the resulting fragments; these are annealed to a slide via the adapters and then amplified by PCR using fluorescently labelled nucleotides with terminators. For each cycle the slide is imaged, the terminators and fluorescent signal is removed and the next labelled base is added. This allows a library to be built up and gives very reliable sequence data (Illumina, 2017).

Roche 454 sequencing is similar but the fragments are much longer up to 1kb. The principle is similar to Illumina but the fragments with adapters are annealed to beads rather than a slide. These beads are then placed in individual wells of a slide. Each dNTP is added separately and a signal read after each is added (Roche, 2017).

Ion torrent and ion proton sequencing do not use imaging like the other types. It instead uses the release of hydrogen ions during nucleotide addition as a measuring tool. As with the other types of sequencing the template is cleaved up, this time to 200bp. A single strand is annealed to a single bead and placed on an individual well of a slide. Then, like 454 sequencing, each nucleotide is added separately and consecutively to the slide. The pH is monitored during the release of hydrogen ions and this is how the reaction is measured (ThermoFisher, 2017).

1.5.3.1.2 Digital PCR

Another novel method being developed is digital PCR (dPCR). This is becoming better known and more researched. The principle of digital PCR is to take a diluted sample and place it into reaction chambers that separate down to single molecules of DNA. The chambers are then counted and marked as positive if they contain a molecule and negative if they do not. This allows absolute

quantification but is relatively expensive to perform at present and so quantitative PCR is more favoured (Baker, 2012). Given a few years, dPCR technology will become more affordable and has great promise at present.

Quantitative PCR (qPCR) or real-time PCR is a development of classic end point PCR. It combines the PCR amplification and detection steps using a fluorescent marker. As the PCR reaction progresses and the number of DNA copies increases, the fluorescent signal also increases which, with today's technology is expressed in graph format. If a reaction is unsuccessful fluorescence will not be detected. A dilution series of a target DNA of known quantity can be performed, subjected to PCR cycling and analysed using a qPCR machine. The output is a standard curve which can be used to quantify unknown samples.

1.5.3.1.3 High resolution melt curve analysis

As part of the analysis at the end of a qPCR reaction it is possible to perform a melt curve analysis. This involves incrementally increasing the temperature from low to high. This separates the double DNA strands within the reaction influencing a change in fluorescence that is released from a DNA dye bound to the once double strands of DNA. When the first negative derivative of fluorescence is plotted against temperature, a sharp peak will be evident when nearing the melting point of the DNA product due to the rapid loss of fluorescence detected. Different length strands of DNA have different melting temperatures, and GC content and complementarity of the amplicons is also a factor in the melting temperature. Individual peaks will be shown for the different sized strands as long as the size difference is great enough. In theory an individual peak will be shown for each sized product within a reaction but there are limitations to the technique. There must be a large enough difference between the sizes of the DNA products in order to differentiate the two. This method has been developed into high resolution melt curve analysis (HRM) and is exactly as the name describes, in theory this method is able to differentiate between products of just 1 base pair difference. The dye used in HRM does not have the same pitfalls as the classic SYBR based dyes of classic melt curve

analysis. SYBR based dyes can inhibit PCR reactions at higher concentrations and tend to re-associate with the strands of DNA during melting affecting the end result. The dyes used in HRM are so called 'release on demand' and give a much clearer picture. This method has also been considered for barcoding in place of sequencing and has been termed 'bar-HRM'. The principle is not based on size difference but rather single mutations unique to a particular species which could be detected using HRM. This has been successfully applied to several plant species so far including; *Sideritis* species using ITS2 (Kalivas et al., 2014) and *Panax notoginseng* using the region *trnH-psbA* (Tong et al., 2014), to name a few examples. As mentioned in an earlier chapter mini barcodes can be used to overcome degradation of DNA. This was applied with bar-HRM in the species, *Acanthus ebracteatus*, *Andrographis paniculata*, and *Rhinacanthus nasutus* based on the *rbcL* region (Osathanunkul et al., 2015) and also to *Hypericum perforatum* contamination with *Hypericum androsaemum* using mini *matK* (Costa et al., 2016).

In general, DNA based methods have an advantage over chemical methods as they give an exact and definite identification.

1.5.3.2 PlantID

PlantID is an innovative method developed by researchers at De Montfort University in collaboration with the East Midlands Forensic Pathology Unit. The PlantID project was supported by the Healthcare and Bioscience iNet, which is funded by the East Midlands Development Agency and the European Regional Development Fund.

The original PlantID system was developed using *Hypericum perforatum* L. , more commonly known as St John's Wort, as the target, and resulted in the ability to discriminate between four closely related species of *Hypericum* (Howard et al., 2011).

The PlantID system entails two steps in the working assay. The first is multiplex PCR of a sample with fluorescently labelled primers specific to a number of species of interest respectively. The second is

capillary electrophoresis of the PCR product. The system, once up and running, is easy to carry out and rapid (Howard et al., 2011). The main downfall is the expense of using capillary electrophoresis but could potentially be overcome by using high resolution gel electrophoresis or mini-capillary electrophoresis in place of this. The Lab-on-a-Chip (Agilent) and the QIAxcel (Qiagen) systems are examples that could be used.

The final working PlantID method requires little expertise to perform and analyse, and is reproducible. It is also a rapid technique and fairly cheap to run after capital costs.

1.6 Aims

- To further develop a high specification DNA assay to distinguish Black Cohosh, *Actaea racemosa,* from extremely closely related likely adulterant species in mixed samples. The assay has the ability to detect multiple species in one sample. The number will be increased from 3 species to 5 species.
- To develop a qPCR assay to confirm the identity of commercial Black Cohosh Products.
- To assess the effect of *Actaea racemosa* and two closely related species on human liver cells.

1.7 Objectives

The above aims would be achieved by carrying out the following;

- PCR of *Actaea* species with specific primers. PCR of *Actaea* species with other species specific primers to assess specificity. Optimisation of multiplex PCR and exploration into potential platforms for analysis.
- Apply the above developed test to available commercial products
- Use the species specific primers in qPCR to test for the presence of certain *Actaea* species.

- Apply the qPCR assay to assess a wide range of available products from the UK and American market.

- Treat human liver cell lines with extracts of *A. racemosa* and 2 closely related *Actaea* species.

- Extract RNA, convert to cDNA and analyse using gene expression with a specifically designed qPCR array capable of assessing 84 genes associated with hepatotoxicity.

2 Genetic analysis of Black Cohosh, *Actaea racemosa,* and potential adulterant species

2.1 Introduction

Establishing a library of reference sequences is fundamental in the identification of plants using DNA based methods. The identification of specimens by sequencing relies on comparison to reference sequences from sequence databases. The identification of the material for these reference sequences must be achieved with high confidence. Even samples identified by expert taxonomists can be misidentified so thorough analysis of each sequence is paramount (Coutinho Moraes et al., 2015). According to Chen et al 2014, in order to develop a reliable database of barcode sequences, the following must be achieved: three uniquely labeled duplicate collections of samples for each species with aliquots of material for DNA extraction and whole flowering plants for herbarium voucher samples (Chen et al., 2014b). This is in agreement with Smilie and Khan 2010, with both publications also stating the importance of photographs of the herbals in the environment from which they were harvested, collector's details, detailed location, extensive notes of characters not evident from herbarium sample and for the material to be free of microbial contamination and infestation (Smillie and Khan, 2010). This information would aid a taxonomist in the identification of the raw material. It also makes vouchering of the material more conclusive and credible. Vouchered samples are not always identified correctly, jeopardizing the validity of any future work based on sequencing outcomes (Chen et al., 2014b). The art of taxonomy is becoming a rare career, and the talent is slowly diminishing (Coutinho Moraes et al., 2015). More often than not, the aforementioned ideal of fully documented vouchering is not achieved or even possible to deliver. This is particularly the case with raw material that has been procured processed in some way. These are the cases where the chance of misidentification increases. The barcode sequence data that is collected for databases is intended for use in identification of the species of a sample, and this is the

fundamental reason why it is important to ensure that the correct plant species has been used to generate this information. The efforts of all the research groups that are generating this information are of great value to the Barcode of Life effort.

The parts of the plant genome focused on in this chapter are the Nuclear Ribosomal Internal Transcribed Spacer (nrITS) and the plastid region maturase K (*matK).*

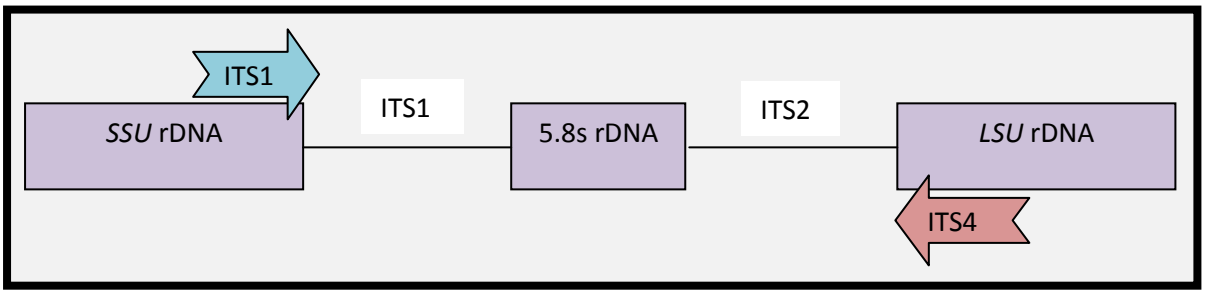


Figure 14: A diagram to show the nrITS region and placement of primers ITS1 and ITS4

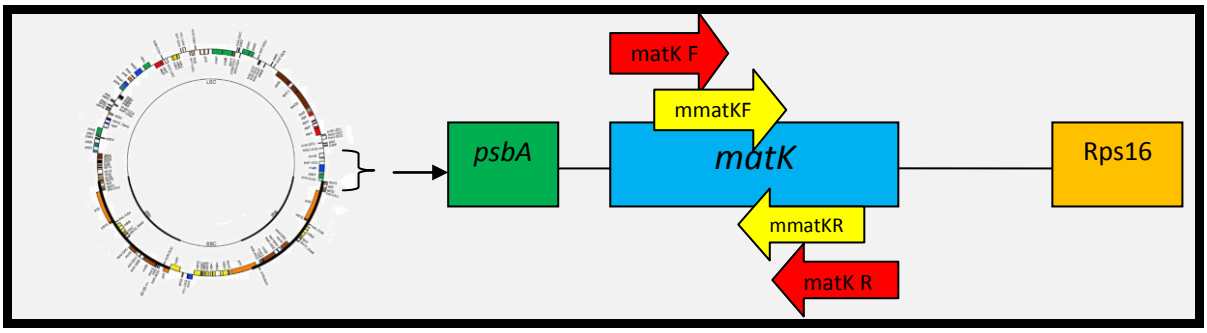


Figure 15: A diagram to illustrate the chloroplast DNA of a plant and where the matK region is located

The nrITS region was chosen as it is a proven barcode region of value. It has been demonstrated in several species to allow discrimination due to unique points in the region. It has been found to reap reproducible results and yield good quality sequence data. In fact ITS2, a smaller portion of the nrITS region, has been identified as a barcode for herbal medicines as well as *trnH-psbA* (Chen et al., 2014b). Due to the success of Baker et al 2012 in using mini *matK* to aid identification of species in commercial products, it was decided to use this region where it was not possible to successfully amplify the ITS region, however this was used with caution due to the lack of sequence entries for some species, for example there are no *matK* sequences for *Actaea heracleifolia.*

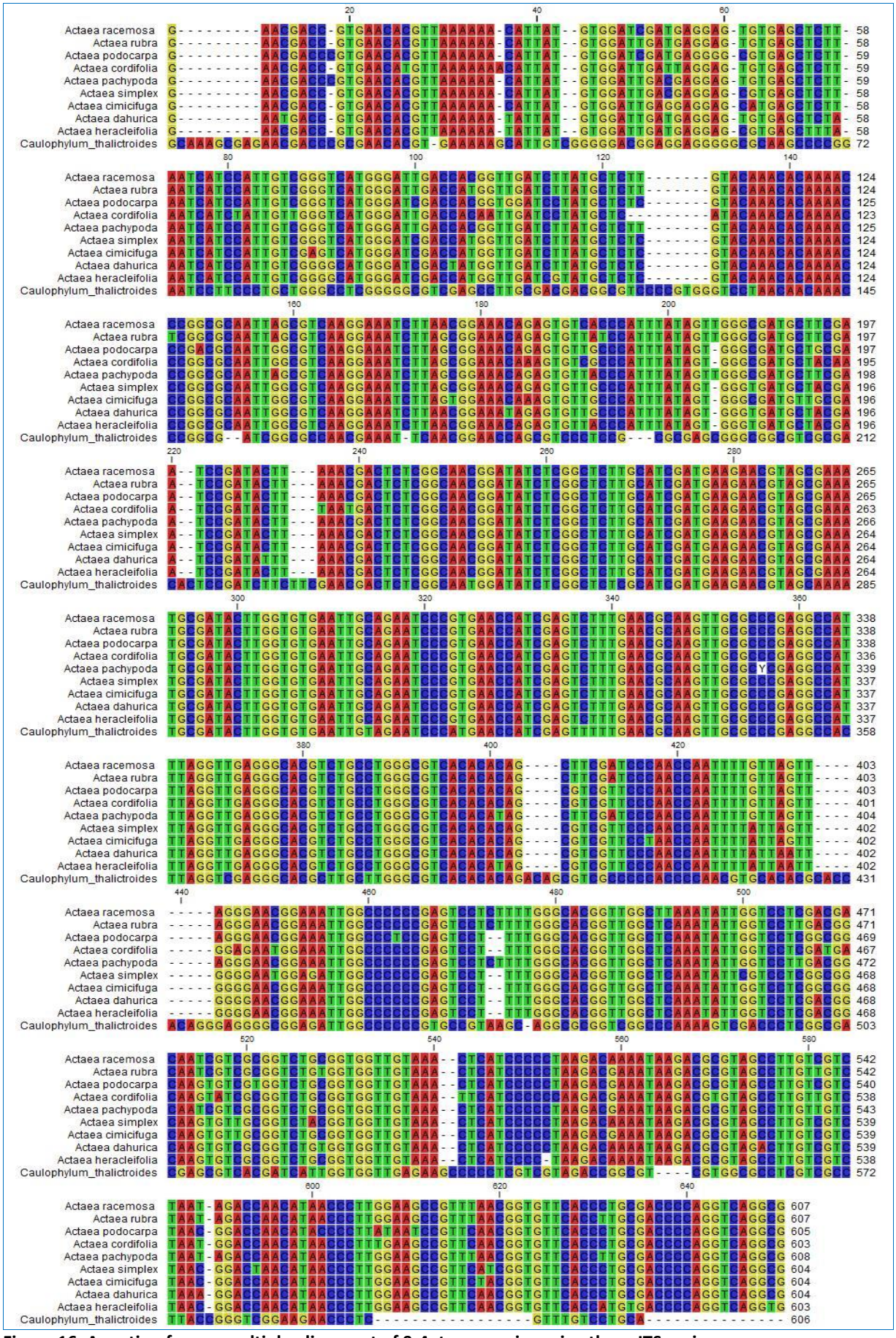


Figure 16: A section from a multiple alignment of 9 *Actaea* species using the nrITS region

Figure 16 displays differences in the genetic code of the nrITS region of the chosen species. This makes it possible to differentiate between the species and allocate identification. *Caulophyllum thalictroides* is from a completely different family to the *Actaea* species and therefore was difficult to align.

Some of the sequence data utilized in this work was obtained from GenBank. GenBank is a freely accessible database of sequence data generated by researchers all over the world. In fact this is where the information was taken from to design species specific primers that were used in the MSc by Research degree project (Williams, 2012). At the time of design, barcoding was a relatively new idea and at the start of development, so sequence data for barcode regions was not in abundance. The *Actaea* sequence data was gathered from the work of Compton et al 1999, and the original purpose of this sequencing effort was to analyse the members of the *Actaea* genus, which at the time were the two separate genera; *Cimicifuga* and *Actaea* (Compton et al., 1998).

The remainder of the sequence data was generated by sequencing vouchered DNA specimens and by also extracting DNA and performing sequencing for samples from many other sources. These sources ranged from plants grown from seeds to samples of roots provided by other research groups. The nrITS region was then amplified using PCR and directly sequenced.

The main outcomes of this chapter are as follows:

- To build up a library of reliable reference sequences for several species from the *Actaea* genus, with focus on the Black Cohosh species, *Actaea racemosa.*
- Construct contigs of sequence data from multiple reads
- Analyse the obtained sequence data of reference samples using BLAST (Basic Local Alignment Search Tool) and by constructing tree diagrams of the top 100 BLAST sequences to show relationships between sequence accessions.

- Confirm identification for the reference samples.

2.2 Materials and methods

2.2.1 Sourcing of plant materials and vouchered DNA samples

Vouchered DNA specimens were the gold standard to be used in the development of the assays in this work, but supply was very limited and vouchers were not available for all the species that were to be investigated. Samples were obtained from many sources; the Royal Botanic Gardens, Kew, from Dr Eike Reich of CAMAG (Muttenz, Switzerland), samples grown from seeds (Beeches Nursery, Saffron Waldon, UK and Secret Seeds, Devon) and samples provided from other researchers such as Dr Maged Sharaf from the AHPA and Dr Richard Middleton of the BHMA.

Table 12: A list of information for reference samples

Species (Implied or known)	Source	DNA bank ID/Sample No.	Type
Actaea racemosa	Kew	24092	DNA extract
Actaea cordifolia	Kew	20113	DNA extract
Actaea pachypoda	Kew	550	DNA extract
Actaea dahurica	Kew	24397	DNA extract
Actaea cimicifuga	Kew	10294	DNA extract
Caulophyllum thalictroides	Kew	28066	DNA extract
Actaea heracleifolia	CAMAG	52785BRM	Powdered root/rhizome
Actaea rubra	CAMAG	52544	Powdered root/rhizome
Actaea podocarpa	CAMAG	53504BRM	Powdered root/rhizome
Actaea heracleifolia	CAMAG	58448	Powdered root/rhizome
Actaea heracleifolia	CAMAG	58449	Powdered root/rhizome
Actaea dahurica	CAMAG	58450	Powdered root/rhizome
Actaea simplex	Beeches Nursery	N/A	Fresh leaf
Actaea cimicifuga	Secret Seeds	N/A	Fresh leaf
Actaea cordifolia	Secret Seeds	N/A	Fresh leaf
Actaea racemosa	Secret Seeds	N/A	Fresh leaf
Actaea pachypoda	Secret Seeds	N/A	Fresh leaf
Actaea racemosa	AHPA	AHPA – BC037	Cut roots
Actaea racemosa	AHPA	AHPA – BC038	Cut roots
Actaea racemosa	AHPA	AHPA – BC039	Cut roots
Actaea racemosa	AHPA	AHPA – BC040	Cut roots
Actaea racemosa	AHPA	AHPA – BC041	Cut roots
Actaea racemosa	AHPA	AHPA – BC042	Cut roots
Actaea racemosa	AHPA	AHPA – BC043	Powdered roots
Actaea racemosa	AHPA	AHPA – BC044	Whole roots
Actaea racemosa	AHPA	AHPA – BC045	Whole roots

Actaea racemosa	AHPA	AHPA – BC046	Whole roots
Actaea racemosa	AHPA	AHPA – BC047	Whole roots
Actaea racemosa	AHPA	AHPA – BC052	Powdered roots
Actaea racemosa	AHPA	AHPA – BC053	Powdered roots
Actaea racemosa	AHPA	AHPA – BC056	Powdered roots
Actaea racemosa	AHPA	AHPA – BC057	Powdered roots
Actaea racemosa	AHPA	AHPA – BC058	Whole roots
Actaea racemosa	AHPA	AHPA – BC059	Extract
Actaea racemosa	AHPA	AHPA – BC061	Whole roots
Actaea racemosa	AHPA	AHPA – BC062	Whole roots
Actaea racemosa	AHPA	AHPA – BC063	Whole roots
Actaea racemosa	AHPA	AHPA – BC064	Cut roots
Actaea racemosa	AHPA	AHPA – BC065	Whole roots
Actaea racemosa	AHPA	AHPA – BC066	Whole roots
Actaea racemosa	AHPA	AHPA – BC067	Whole roots
Actaea racemosa	AHPA	AHPA – BC068	Whole roots
Actaea racemosa	AHPA	AHPA – BC069	Whole roots
Actaea racemosa	AHPA	AHPA – BC071	Whole roots
Actaea pachypoda	AHPA	AHPA – BC078	Whole roots
Astilbe biternata	AHPA	AHPA – BC083	Whole roots
Astilbe biternata	AHPA	AHPA – BC084	Whole roots
Serratula chinensis	AHPA	AHPA – BC091	Cut roots
Actaea dahurica	SynoPhtyo	Ch.B.01230952	Sliced root
Actaea cimicifuga	SynoPhtyo	Ch.B.13884K116-A	Sliced root
Actaea racemosa	Staffort, DHU	DR10-014-A	Whole dried root and rhizome
Actaea racemosa	Staffort, DHU	-	Fresh Leaf
Actaea spicata	Staffort, DHU	-	Fresh Leaf
Caulophyllum thalictroides	Staffort, DHU	-	Fresh Leaf
Actaea dahurica	BGBM DNA Bank	8499	DNA extract
Actaea pachypoda	BGBM DNA Bank	8495	DNA extract
Actaea simplex	BGBM DNA Bank	8498	DNA extract
Actaea rubra	BGBM DNA Bank	8500	DNA extract
Actaea heracleifolia	BGBM DNA Bank	6700	DNA extract

2.2.2 Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit

The Qiagen DNeasy Plant Mini Kit (Qiagen Inc.,CA) and TissueLyser procedure was followed. To a 2 mL safe-lock centrifuge tube, 100 mg of wet plant material or 20 mg of dry material was added. A 3mm tungsten carbide bead was put inside the tube. The tubes were then placed into the TissueLyser adapter set, fixed to the clamps and ground for one minute at 30 Hz. The position of the tubes was then reversed and the previous step was repeated to ensure equal treatment. Then 400 µL of buffer AP1 and 4 µl of RNase A stock solution was added to each sample and mixed thoroughly. To lyse the cells, the samples were then incubated for 10 minutes at 65°C. The samples were mixed 2 to 3 times during the incubation by inversion.

The samples were incubated on ice for 5 minutes after the addition of 130 µL of buffer AP2. This was carried out to precipitate unwanted substances such as polysaccharides and detergent. The tubes were centrifuged for 5 minutes at 20,000 x g to remove the precipitates from the supernatant. The supernatant was next pipetted into a QIAshredder Mini spin column placed inside of a 2 mL collection tube, and centrifuged at 20,000 x *g* for 2 minutes. The resultant flow through fraction from the previous step was transferred to another tube, the volume was noted and 1.5 times volume of buffer AP3/E was added. The samples were then mixed by pipetting.

To a DNeasy Mini spin column, 650 µL of the mixture from the previous step was added and centrifuged for 1 minute at 6000 x *g*, the flow through was discarded. After this step, the rest of the mixture was added to the DNeasy Mini spin column and centrifuged for 1 minute at 6000 x *g*. This flow through was also discarded. The Mini Spin column was placed into a new collection tube and 500 µL of buffer AW was applied. The column was then centrifuged for 1 minute at 6000 x *g* and the flow through was discarded. Another 500 µL of buffer AW was applied to the column and centrifuged this time for 2 minutes at 20,000 x g to dry the membrane, the flow through was again discarded. The mini spin column was carefully transferred to another microcentrifuge tube and 100 µL of buffer AE was pipetted directly onto the membrane of the column. The columns were left to incubate for 5 minutes at room temperature and then centrifuged at 6000 x *g* for 5 minutes. This step was carried out to elute the DNA from the membrane and so the flow though was kept. This step was repeated and the eluates combined.

2.2.3 DNA clean up using isopropanol method

An isopropanol clean-up was used to remove contaminants and also to increase the concentration of the DNA if required. For 50 µL of DNA sample in Tris-EDTA (TE) buffer (pH 8.0), 35 µL of isopropanol was added and the solution was mixed. The solution was then centrifuged for 30 minutes at 15,000 × *g* at a temperature of 4°C. The resulting supernatant was removed and the pellet was re-suspended in 200 µL of ethanol. This was centrifuged for 10 minutes at 15,000 × g at room temperature. The

supernatant was removed and the remaining ethanol was left to evaporate for approximately 20 minutes at room temperature. Finally the pellet was re-dissolved in 50 µL of TE buffer.

2.2.4 Basic DNA clean using the Qiagen DNeasy Mini Kit

DNA samples from Royal Botanical Gardens, Kew contained residual ethidium bromide and caesium salts. Purification was achieved by using a part of the protocol for DNA extraction using the Qiagen DNeasy® Plant Mini-kit, with a few alterations. To begin with, 5 µL of the starting material and 15 µL of AP3/E buffer (1M Guanidine Hydrochloride (4.78 g in 50 mL 100% EtOH)) were placed in a 1.5 mL tube and mixed by vortexing. This solution was then transferred into a DNeasy Mini Spin column (white) placed in a 2 mL collection tube and centrifuged for 1 minute at ≥ 6000 x *g*. The flow through was discarded and the collection tube reused for the next step. To the spin column, 400 µL of Buffer AW, was added and centrifuged for 1 minute at 6000 x *g*. The flow through was discarded and the collection tube reused for the next step. The previous step was repeated but centrifuged for 2 minutes at 20,000 x *g* to dry the membrane of the column. The column was then transferred to a 1.5 mL tube and 25 µL of buffer AE was applied directly onto the membrane. An Incubation step at room temperature was carried out for 5 minutes and then the column was centrifuged for 1 minute at 6000 x *g* to elute. This step was repeated to result in a total elution volume of 50 µL.

2.2.5 Standard (End-point) Polymerase Chain Reaction

For experiments using the standard polymerase chain reaction, a ready-made mix was used called Red Mix (Bioline, London). This mix contains DNA polymerase, dNTPs, $MgCl_2$, and DNA loading buffer already in the correct proportions. The addition of nuclease free water, primers and a template completed the recipe. Reactions were typically 25 µL final volume except if a reaction was being prepared for sequencing and 50 µL would be made. An extra reaction would be included in the master mix to compensate for loss during pipetting. I.e. if 10 reactions were needed the master mix would be made up for 11.

Table 13: The recipe required for each PCR reaction when using Red Mix, Bioline.

Component	Amount for 1 reaction (µL)
2 X Red Mix	12.5
Forward Primer	0.5
Reverse Primer	0.5
Template	1
Nuclease free water	10.5

2.2.6 Gel Electrophoresis using agarose

Agarose gel electrophoresis was used to analyse the results of PCR products. A 1 X Tris/Borate/EDTA (TBE) buffer (pH 8.0) was used in this case for gel electrophoresis. Various gel tanks were used with different capacities. Generally 50mL of TBE and a variable amount of agarose were used to prepare the gel. The agarose powder was added to the TBE and melted using the microwave, which was typically around 90 seconds. To decide upon the percentage of gel required, Table 14 was consulted;

Table 14: Required Agarose gel percentage depending upon amplicon size.

Expected Product Size	Agarose Concentration (w/v)
< 150 bp	3%
150 - 300 bp	2%
> 300 bp	1%

To allow detection of DNA amplicons, 1 µL of SYBR® Safe dye (Invitrogen, Carlsbad, CA, USA), was added to the melted solution. This was then poured into a gel mould with combs in place to create wells and left to solidify. Once set, the combs were removed, the gel was placed into the tank and TBE buffer was poured to the fill level. Typically, 5 µl of PCR product was loaded into each well and 100 V were passed through for 25 minutes. Different voltages and times were required for different products. Generally short amplicons were run on a high voltage for a short amount of time. For reactions containing multiple products, i.e. multiplex PCR, gels were run at a low voltage and long length of time to optimise resolution of products.

The gels were photographed using a BioRad (BioRad, Hercules, California, United States) illuminator with a ChemiDocXRS Camera and Quantity One Software.

2.2.7 DNA sequencing

Sanger sequencing was used and was performed externally (Macrogen, Amsterdam). Samples of good quality, i.e. amplified with ITS primers and showed a bright crisp band on a gel, was sent to the sequencing provider. PCR product was sent to the company who cleaned it up using on column technique and sequenced using the Applied Biosystems ABI3730XL genetic analyzer. This machine has 96 capillaries and is designed to be suited for high throughput applications. The sequence information is then uploaded to an online portal where it is available for the customer.

2.2.8 Bioinformatics tools

Basic Local Alignment Search Tool (BLAST) from NCBI was used to search through the GenBank database of sequences. BLAST is available from the following web address: https://blast.ncbi.nlm.nih.gov/Blast.cgi. The software aligns the query sequence with sequences from the database and returns a list of the top 100 closest matches.

Trees were constructed using MUSCLE to align the sequences, Gblocks to curate the sequences, PhyML to construct the tree and TreeDyn to visualise the constructed tree. These tools were available to use from www.phylogeny.fr (Dereeper et al., 2008, Dereeper et al., 2010).

2.3 Results and discussion

2.3.1 Creation of a Reference Library of reliable database sequences

2.3.1.1 Analysis of available sequence data on GenBank

The reliability of the sequence information used from GenBank was very important in order to be able to identify samples in this work. This data therefore had to be analysed before confidence could be given to any identification. A search was carried out on GenBank for each of the species of interest. For some of the *Actaea* species included, there was a plethora of available information, for others the data was very limited. Table 15 shows this information.

Table 15: The number of sequence data accessions for the ITS region for species of interest in this project

	Region	
Species	***matK***	**nrITS**
Actaea racemosa	53	29
Actaea dahurica	5	49
Actaea cimicifuga	7	25
Actaea heracleifolia	0	22
Actaea rubra	17	10
Actaea cordifolia	2	4
Actaea simplex	8	150
Actaea pachypoda	15	8
Actaea podocarpa	6	7
Caulophyllum thalictroides	5	2

(*Correct as of 06.03.2017*)

For nrITS, the sequences were aligned and then organised into a tree diagram to assess which of them were reliable to use. This is based on the concept of 'DNA taxonomy' described by Blaxter 2004. It is important to note that this is not a phylogenetic tree. The purpose of the tree diagrams in this chapter is not to show evolutionary relationships, but rather to show how similar the sequence data is. The sequences that are grouped together in a 'tree branch' are termed as molecular operational taxonomic units or MOTU, and this visually displays similarity and therefore whether the sequences belong to the same species. This was an effective way to quickly identify the best

sequences in terms of reliability (Blaxter, 2004). If the top sequence hit from BLAST, in other words the best matched sequence from GenBank, was grouped into a 'tree branch' with other sequences of the same labelled species, this would be a reliable identity. Some of the *Actaea simplex* sequences had to be omitted from the diagram as the software has a limit of 200 sequences and there was many for this species.

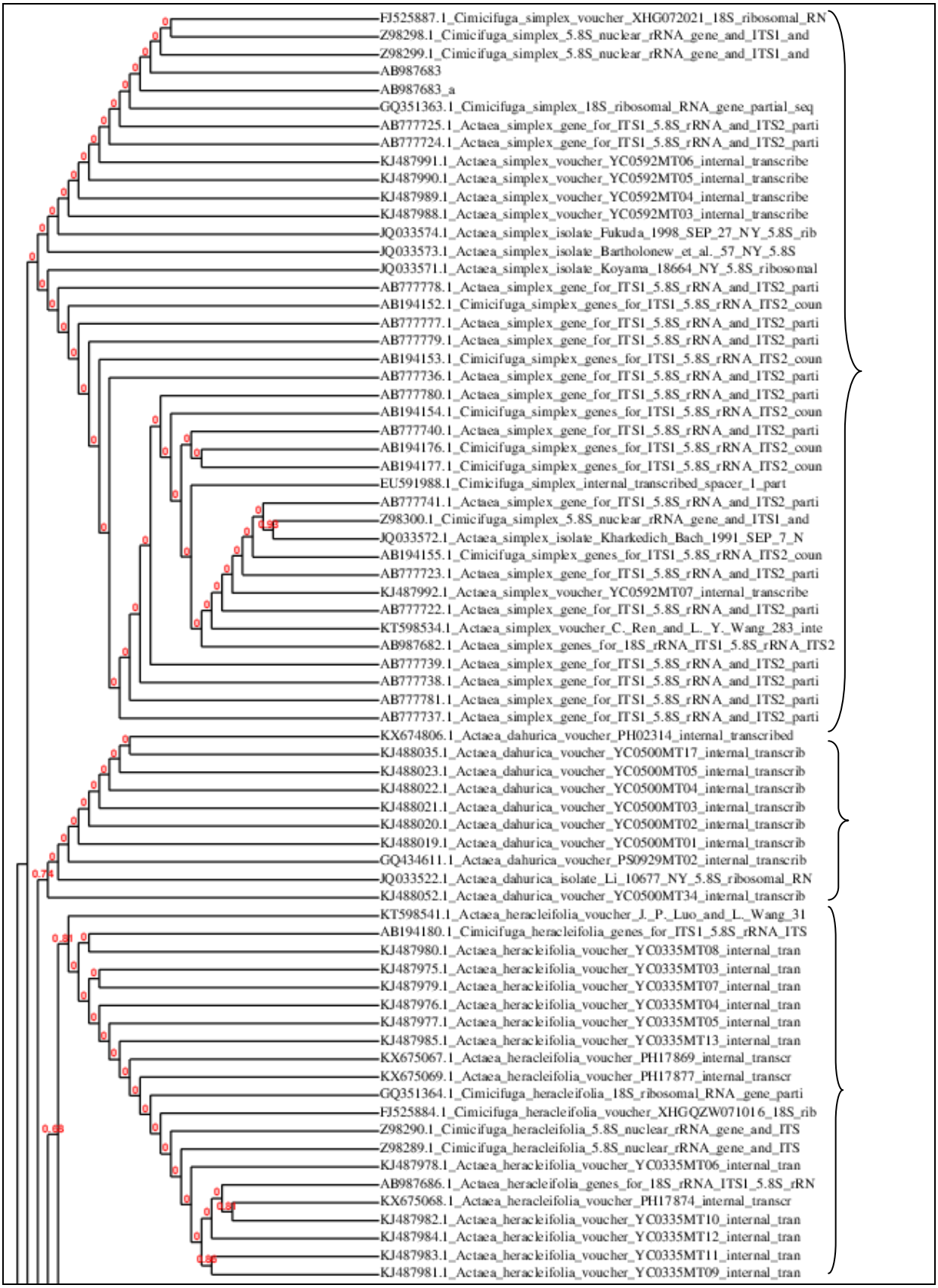


Figure 17: A portion of tree diagram displaying available *Actaea* nrITS sequences from GenBank – portion 1

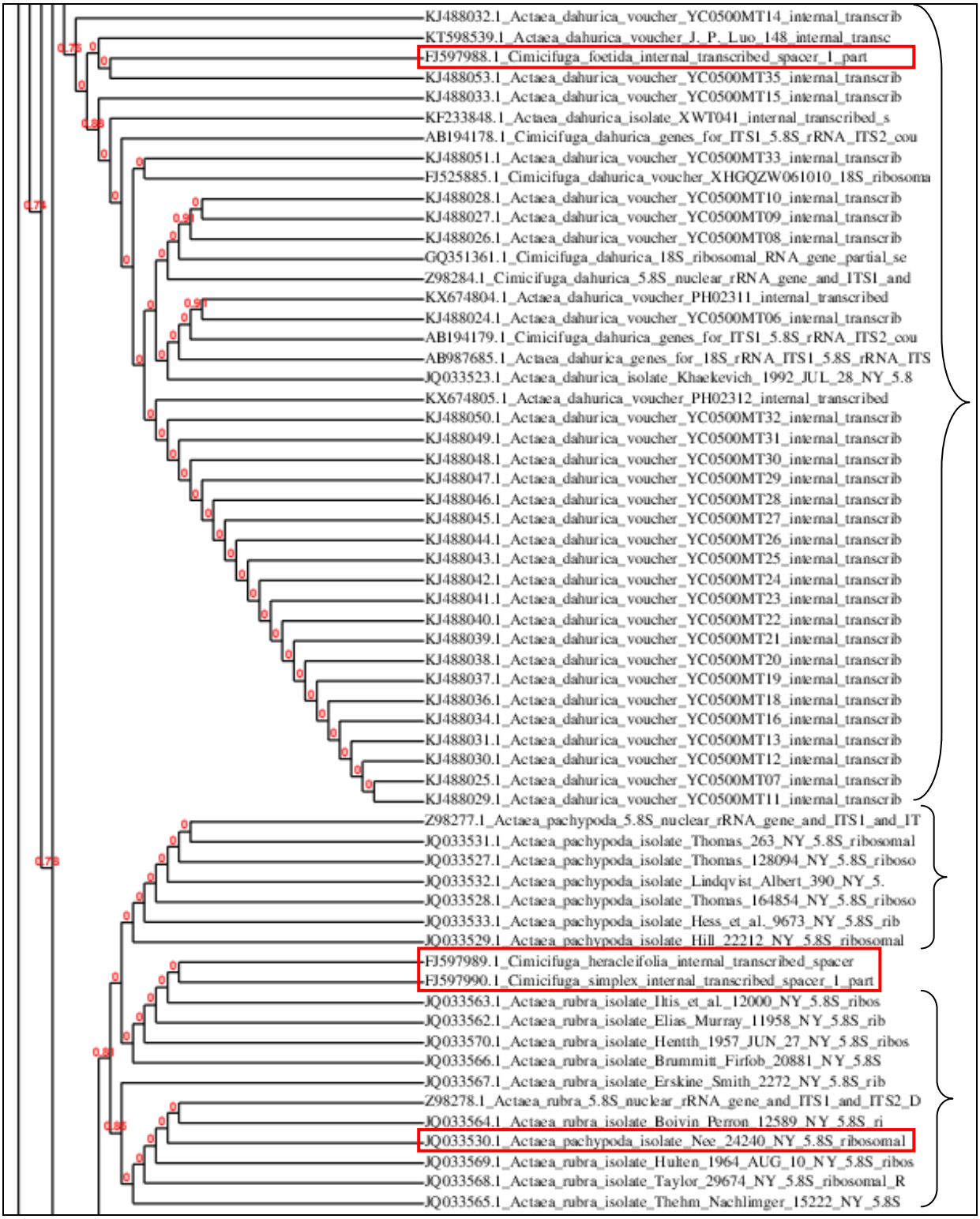


Figure 18: A portion of tree diagram displaying available *Actaea* nrITS sequences from GenBank – portion 2

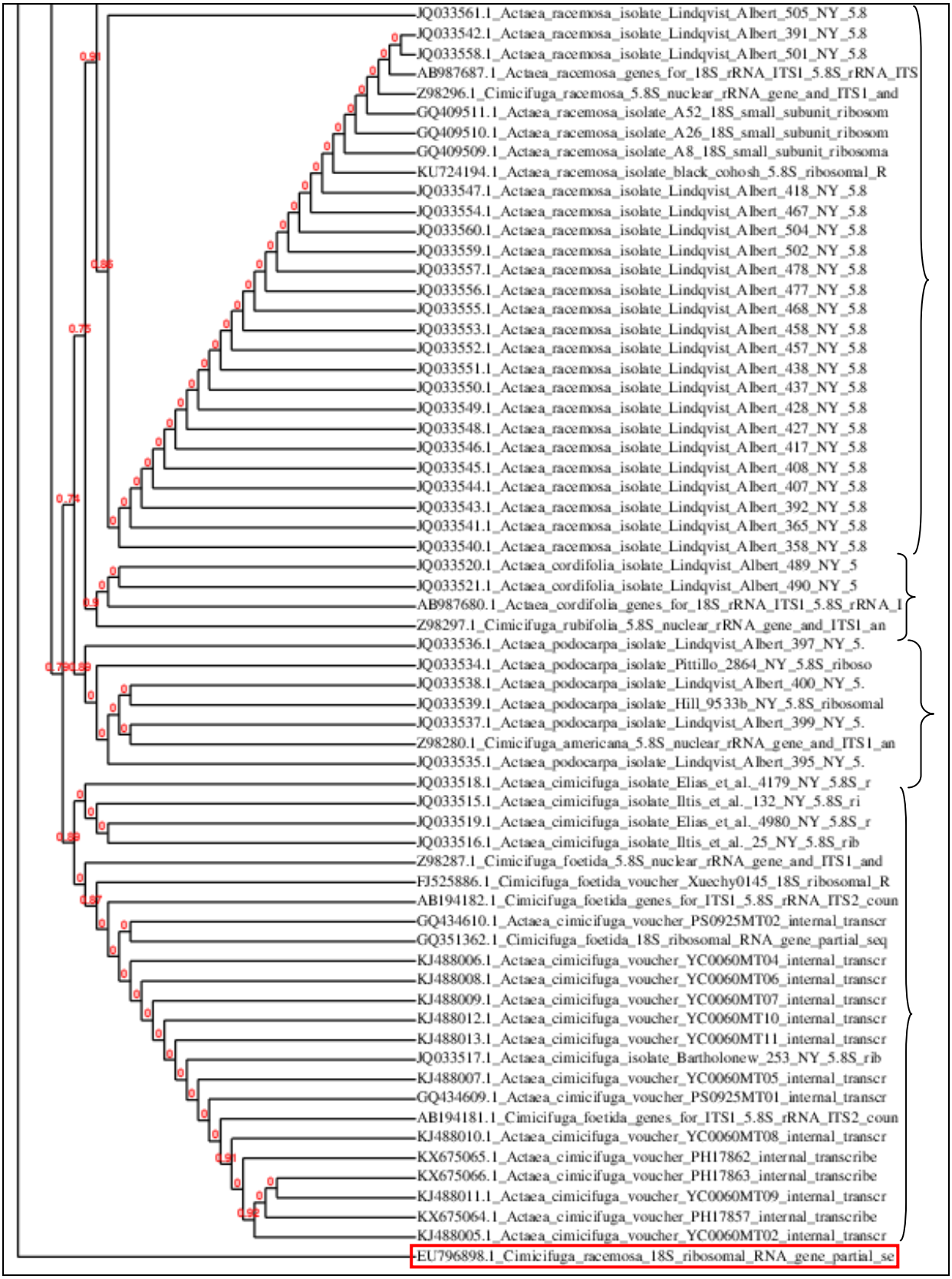


Figure 19: A portion of tree diagram displaying available *Actaea nr*ITS sequences from GenBank – portion 3

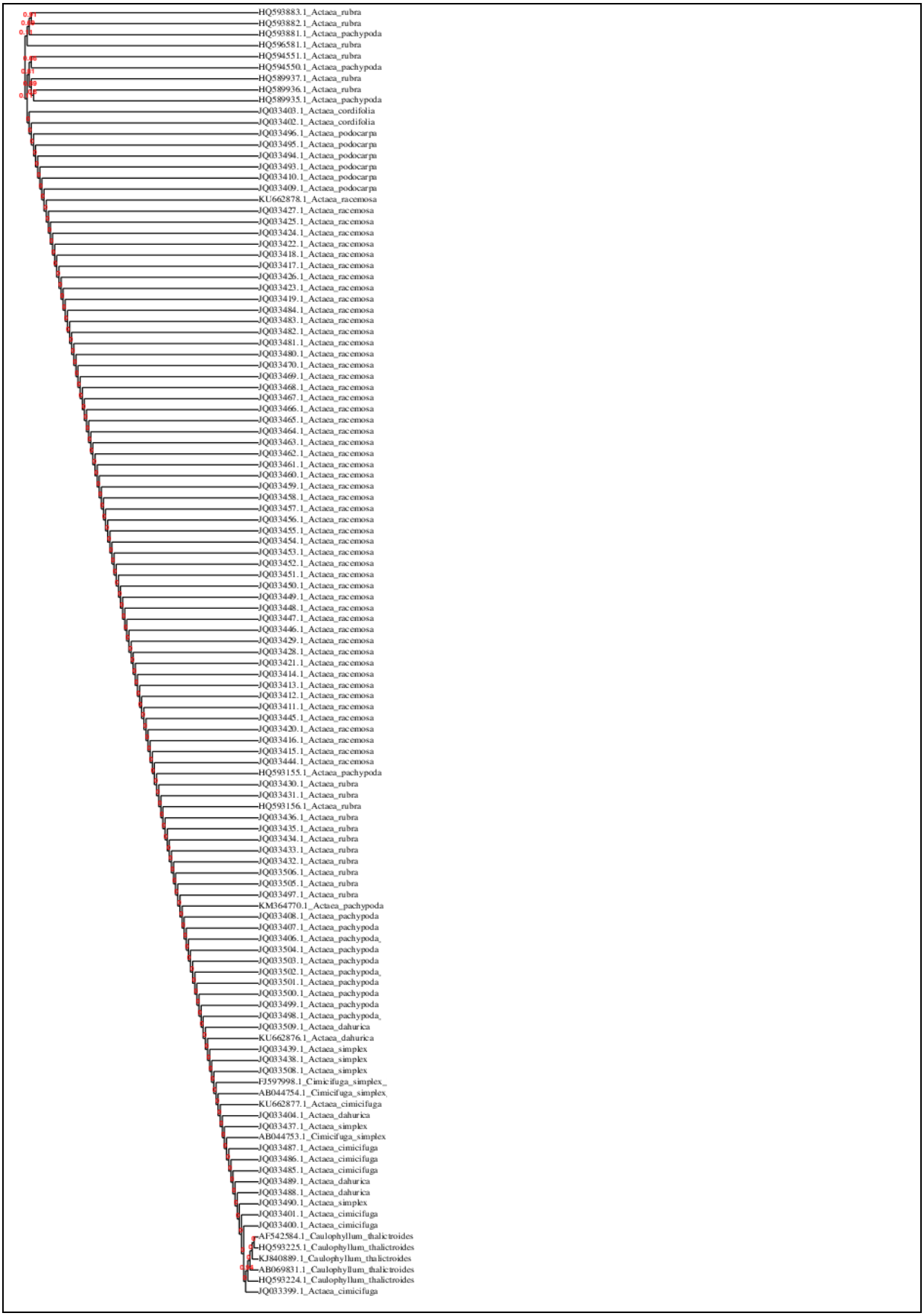


Figure 20: A tree diagram of available sequences for the *MatK* region of Actaea species and *Caulophyllum thalictroides.*

For nrITS most of the entries were clearly grouped into branches with species of the same name. These sequences were deemed reliable to use for identification of own sequence data. There were some odd entries that did not get grouped in a branch of the same species. If there were any matches to these sequences then this identification would not be seen as reliable as they are highly likely to have been misidentified.

For mini-*matK* the available sequence entries (Shown in Figure 20) were not grouped at all and for this reason this region would not be used alone to identify samples. This region would be used if the nrITS could not be amplified and would only be used in combination with results from other methods.

2.3.1.2 Identification of reference sample collection using the nrITS and matK region

Reference samples were collected from many sources. DNA samples from vouchered specimens were obtained from Kew Royal Botanical Gardens (RBG), London and also from the Botanical Garden and Botanical Museum (BGBM), Berlin. Samples were also obtained from collaborations with other researchers from the American Herbal Products Association (AHPA), British Herbal medicines Association (BHMA) and CAMAG, Switzerland. Finally there were some samples from material suppliers that were of interest to identify for future applications.

The DNA samples from RBG, Kew had to be cleaned up as there were PCR inhibitors present from the DNA extraction method. It was initially found that PCR was unsuccessful without a DNA clean up step. The DNA samples from BGBM, Berlin were ready to use. The rest of the samples arrived in a form that required DNA extraction. Once this was achieved all the samples were amplified using PCR with ITS primers. Where a good product with ITS primers could not be obtained, mini *matK* primers were used instead, but as mentioned this was with caution and only to back up other results. This information was never used alone for identification. The PCR products were sent to an external sequencing provider and results were uploaded to a portal for download.

The sequences when obtained were first checked for quality. A good quality sequence has good strongly signalled single peaks and clearly defined base pair demotions for each peak.

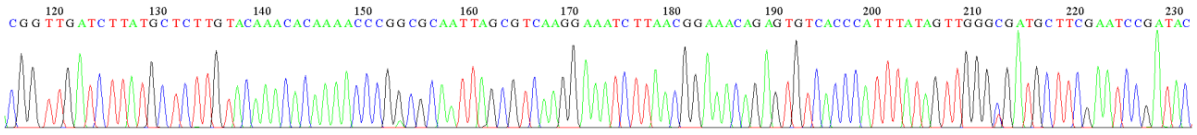


Figure 21: A section of a good quality sequence trace – showing good intensity clear peaks

Figure 21 displays a section of a good quality sequence. Clear well defined peaks are displayed and each peak has been designated a DNA base.

Usually three reads of a sample are performed; two with the forward primer and one with the reverse primer, and this was carried out when a sequence did not match well to a reliable GenBank sequence. If a first read was a good match to a sequence from GenBank and the match was assigned to a MOTU with the same species, then this read was deemed enough to confirm identification.

In the cases where multiple reads were performed, contigs were assembled. This involved aligning the three reads and then systematically resolving any conflicts of base pair denotation. The sequences are also trimmed to remove poor quality sections from the start and/or end of the sequence.

All of the sequence traces supplied by the sequencing providers can be found in the appendix section.

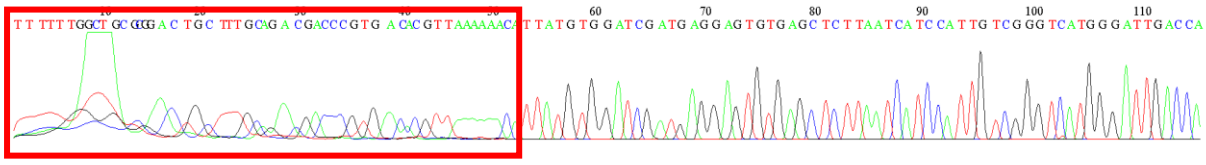


Figure 22: A section of the start of a sequence trace to illustrate poor quality areas that would be trimmed

The sequence trace in Figure 22 shows the beginning of the data and the part in the red box would be trimmed off. The peaks are not clearly defined and in places there is multiple peak detection. This

would affect how well a sequence could be matched to one from a database and so must be attended to.

Next the sequences were identified using information from GenBank. This was possible by using BLAST to search through the GenBank database. The software aligns the input sequence with the available sequences on the Genbank database and returns the top 100 closest matches. It also gives a percentage match and coverage percentage. Each sequence accession that a query sample was matched to was checked for which MOTU it was assigned to. In general if a sequence match was grouped with other of the same species in a MOTU, the result was considered reliable.

Table 16 shows a summary of each of the reference samples that were analysed. The table shows the original species labelling, the identification from BLAST and where the BLAST match sequence is organised in the DNA tree. This information will be useful in the later chapters where assays are developed and validated with some of these samples. Samples where identification has been confirmed can be used. Samples that have had another identification confirmed other than how the sample was labelled could be used for validation based on the sequencing information. Some of these sequences can be added to the databases to broaden what is available. This will be a very valuable addition.

Table 16: Results of BLAST searching for sequenced samples – top hits shown.

Extraction number	Sample number	Source	Sample Type	Species Label	Blast search top hit	Accession number	% ID to top match	MOTU
BC046	Ch.B.13884K1 16-A	Synophyto	Bulk Raw Material for making extracts	*A .cimicifuga*	*A. dahurica* or *A. cimicifuga*	GQ351361.1 JQ033518.1	81% 87%	*A. dahurica* *A. cimicifuga*
BC050	DR10-014-A	Staffort, DHU	Bulk Raw Material for making extracts	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
135	53504BRM	CAMAG	Powdered Roots	*A .podocarpa*	*A. podocarpa*	Z98280.1	99%	*A. podocarpa*
157	52784BRM	CAMAG	Powdered Roots	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
158	52483BRM	CAMAG	Powdered Roots	*A. pachypoda*	*A. pachypoda*	Z98277.1	99%	*A. pachypoda*
161	52789BRM	CAMAG	Powdered Roots	*A .dahurica*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
BC052	58448	CAMAG	Powdered Roots	*A. heracleifolia*	*A. dahurica*	GQ351361.1	99%	*A. dahurica*
BC053	58449	CAMAG	Powdered Roots	*A. heracleifolia*	*A. dahurica*	JQ033523.1	96%	*A. dahurica*
132	52544	CAMAG	Powdered Roots	*A. rubra*	*A. rubra*	Z98278.1	99%	*A. rubra*
-	550	Kew	DNA Extraction	*A. pachypoda*	*A. pachypoda*	Z98277.1	99%	*A. pachypoda*
-	24092	Kew	DNA Extraction	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
-	24397	Kew	DNA Extraction	*A. dahurica*	*A. simplex*	AB987682.1	99%	*A. simplex*
-	10294	Kew	DNA Extraction	*A. cimicifuga*	*A. cimicifuga*	GQ351362.1	99%	*A. cimicifuga*
-	20113	Kew	DNA Extraction	*A. cordifolia*	*A. cordifolia*	AB987680.1	99%	*A. cordifolia*
080	-	Beeches nursery	Fresh Leaves	*A .simplex*	*A. simplex*	AB987683.1	99%	*A. simplex*
067	-	Secret Seeds	Fresh Leaves	*A. dahurica*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
068	-	Secret Seeds	Fresh Leaves	*A. cimicifuga*	*A. cimicifuga*	GQ351362.1	98%	*A. cimicifuga*
069	-	Secret Seeds	Fresh Leaves	*A. cordifolia*	*A. cordifolia*	AB987680.1	99%	*A. cordifolia*
070	-	Secret Seeds	Fresh Leaves	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
071		Secret Seeds	Fresh Leaves	*A. rubra*	*A. rubra*	Z98278.1	99%	*A. rubra*
072	-	Secret Seeds	Fresh Leaves	*A. pachypoda*	*A. pachypoda*	Z98277.1	99%	*A. pachypoda*
S12	AHPA – BC064	AHPA	Whole roots	*A. racemosa*	*A. racemosa*	GQ409511.1	98%	*A. racemosa*
S13	AHPA – BC065	AHPA	Whole roots	*A. racemosa*	*A. racemosa*	GQ409509.1	98%	*A. racemosa*
S14	AHPA – BC068	AHPA	Whole roots	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*

S15	AHPA – BC078	AHPA	Raw Material Roots	*A. pachypoda*	*A. pachypoda*	Z98277.1	99%	*A. pachypoda*
S22	AHPA – BC053	AHPA	Powdered Roots	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
S25	AHPA – BC058	AHPA	Whole roots	*A. racemosa*	*A. dahurica*	AB987685.1	98%	*A. dahurica*
1119	AHPA – BC044	AHPA	Whole roots	*A. racemosa*	*A. cimicifuga*	GQ351362.1	99%	*A. cimicifuga*
1116	AHPA – BC039	AHPA	Root and Rhizomes	*A. racemosa*	*A. racemosa*	AB987687.1	99%	*A. racemosa*
S21	AHPA – BC052	AHPA	Powdered roots	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
S8	AHPA – BC042	AHPA	Root and rhizomes	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
S9	AHPA – BC043	AHPA	Powdered root/rhizome	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
S17	AHPA – BC066	AHPA	Whole roots	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
S18	AHPA – BC067	AHPA	Whole roots	*A. racemosa*	*A. racemosa*	AB987687.1	95%	*A. racemosa*
1117	AHPA – BC040	AHPA	Root and rhizomes	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
BC055	-	Staffort, DHU	Fresh Leaves	*A. racemosa*	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
BC056	-	Staffort, DHU	Fresh Leaves	*A. spicata*	*A. spicata*	Z98279.1	99%	Species not included in tree
BC057	-	Staffort, DHU	Fresh Leaves	*C. thalictroides*	*C. robustrum*	JX040540.1	98%	*N/A – non-Actaea*
-	8495	BGBM	DNA Extraction	*A. Pachypoda*	*A. pachypoda*	Z98277.1	98%	*A. pachypoda*
-	8498	BGBM	DNA Extraction	*A. simplex*	*A. simplex*	AB987682.1	99%	*A. Simplex*
-	8499	BGBM	DNA Extraction	*A. dahurica*	*A. dahurica*	GQ351361.1	99%	*A. dahurica*

The sequence data for the SynoPhyto material was not of the best quality therefore it was not possible to obtain a high percentage match. As a guide, anything less than a 95% match does not give a reliable identification, as there are several differences in the sequences at this point.

The identification for the material labelled as *Caulophyllum thalictroides* is also not firm as there were limited sequence entries to obtain a match from. There were just two entries, one for ITS1 and one for ITS2 which are separate parts of the nrITS region. The sample BC057 was also a match for *Caulophyllum thalictroides* at 98% but much smaller coverage due to the sequence entry length being much smaller than the query sequence. In Figure 23, an alignment of the two matches and the query sequence are displayed. It is shown in this figure that there are around the same number of differences between the query sequence to each of the matches respectively. This leaves a level of uncertainty and this is mainly due to the lack of sequence information on GenBank for this species.

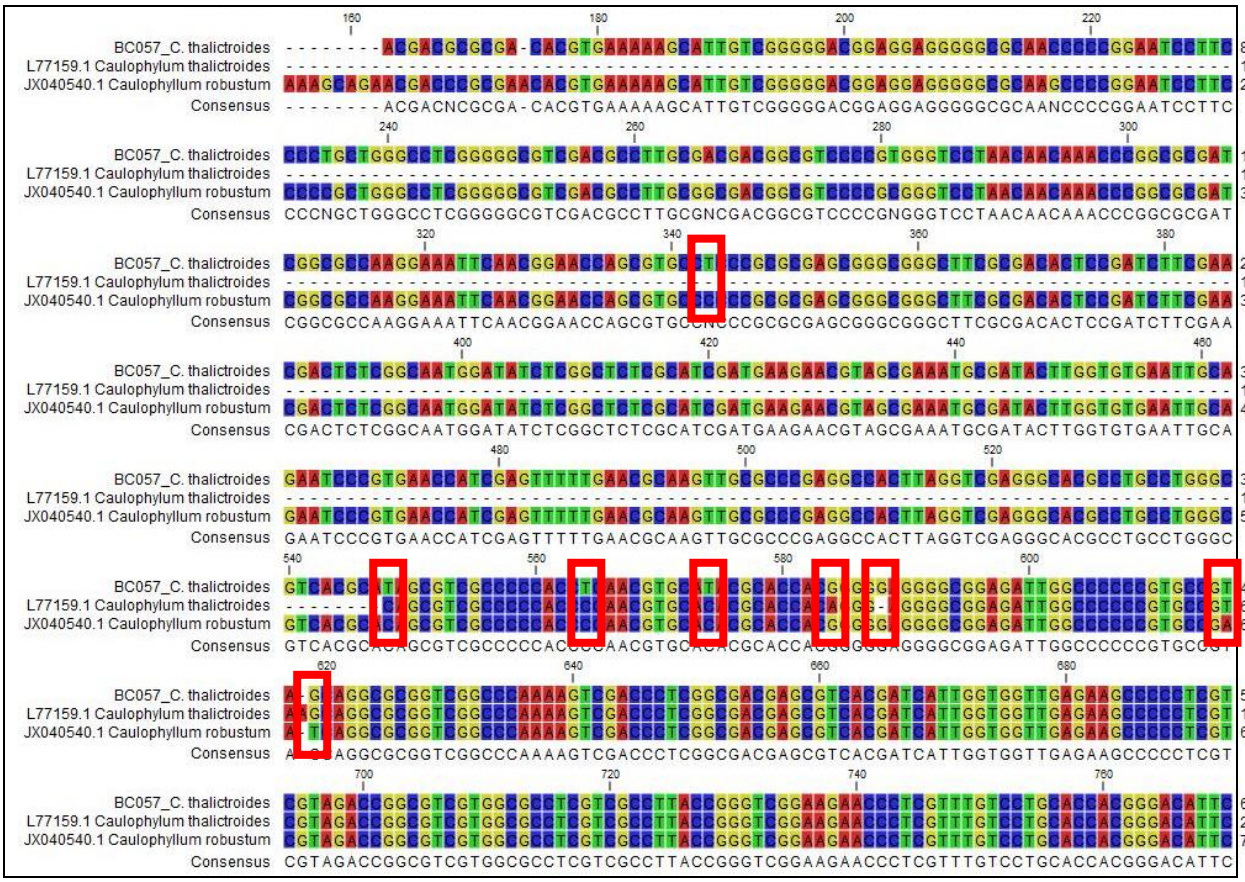


Figure 23: An alignment of BC057 – *C. thalictroides* with two potential matches from GenBank – red boxes show differences between the sequences.

There were some other samples that were not in agreement with the labelled species to the sequencing identification. One sample from the AHPA was labelled as *Actaea racemosa* but was actually sequenced to be *Actaea cimicifuga.* A few of the powdered samples from CAMAG were also sequenced to be different from the labelled identification and one of the plants grown from seeds was identified incorrectly.

One of the DNA extracts from Kew was identified to be *Actaea simplex* rather than *Actaea dahurica.*

The rest of the samples had their labelled identifies confirmed with sequencing and will make useful additions to the sequencing data available along with being reliable samples for assay development and validation samples.

2.4 Conclusions

Overall it was possible to show that the majority of the GenBank sequences for the nrITS region of *Actaea* were reliable. The tree diagrams were able to show that most of the sequences had the correct species assigned to them as these sequences were correctly grouped together. It would be useful to the research community to add the sequences obtained in this chapter to what is available to broaden the number. Particularly for the species which have limited accessions. *Caulophyllum thalictroides* requires more entries in the database. Although there are two entries, they are two separate parts of the nrITS region, one being ITS1 and the other being ITS2.

The matK region is not reliable to use alone as there is not enough difference to completely separate the species in the Actaea genus. This region would only be used if it is not possible to amplify the nrITS region when assurances are needed from sequencing to back up other results. They will still be used with caution however.

This is the first time an appraisal of the available sequence data has been completed and the fact that the majority of the data has been shown to be reliable is a great contribution to the scientific community.

2.4.1 Sequencing of raw material from SynoPhyto, China and Staffort, Germany

The *A. cimicifuga* material purchased from SynoPhyto was intended to manufacture ethanol extracts along with material identified as *A. dahurica.* The *A. dahurica* material is not mentioned in the summary as it was not possible to obtain a PCR product from the ITS region. This could be for a number of reasons including DNA degradation due to processing techniques. It was important to attempt to identify these materials as the resulting extracts were intended for treatment of cultured human hepatocytes and assessment of hepatotoxicity (Chapter 5. Investigation into potential hepatotoxicity of three *Actaea* species). The *A. racemosa* material obtained from Staffort, DHU, was also intended for extract production and so was of equal importance to identify. The *A. cimicifuga* material was not clearly identified using sequencing. The sequence data was not of high quality and so it was not possible to match with GenBank sequences with confidence. The trace obtained from the sequencing provider showed a low intensity.

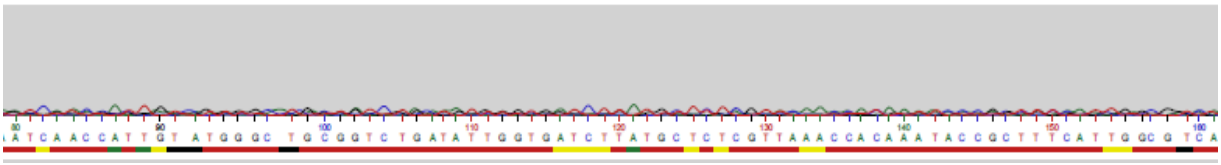


Figure 24: A portion of the sequence trace from the *A. cimicifuga* material from SynoPhyto

The blast search showed *Actaea dahurica* as the top hit at 81% as it had the most coverage of 91% of the sequence. *Actaea cimicifuga* was coming up as a match further down the list of hits, with several showing as 87% identification with less coverage of the query sequence (48-51%). When the top 100 hits from BLAST are organised into a tree diagram it can be shown that there is very little relationship between any of the hits thus reinforcing the bad quality of the sequencing data. This is displayed in Figure 25.

Figure 25: A tree diagram of the top 100 hits for *A. cimicifuga* material from SynoPhyto – Query sequence and top hit framed in red.

For this example it was necessary to look a bit deeper at the alignments. These are shown in Figure 26. Both hits have many discrepancies and therefore this data is inconclusive. When the two different sequences are aligned, *Actaea dahurica* and *Actaea cimicifuga*, it can be seen that they are themselves very similar. This material will also be assessed with a qPCR assay in a later chapter. The PCR product in the qPCR assay was designed to be smaller than the ITS PCR product and so there was the potential to obtain a better quality product with this method and overcome any potential degradation (Chapter 4 Development of a qPCR assay for the authentication of *A. racemosa* and detection of potential adulterants). A HPTLC assay would also be used to assess chemical constituent patterning along with a taxonomist assessment of the raw material.

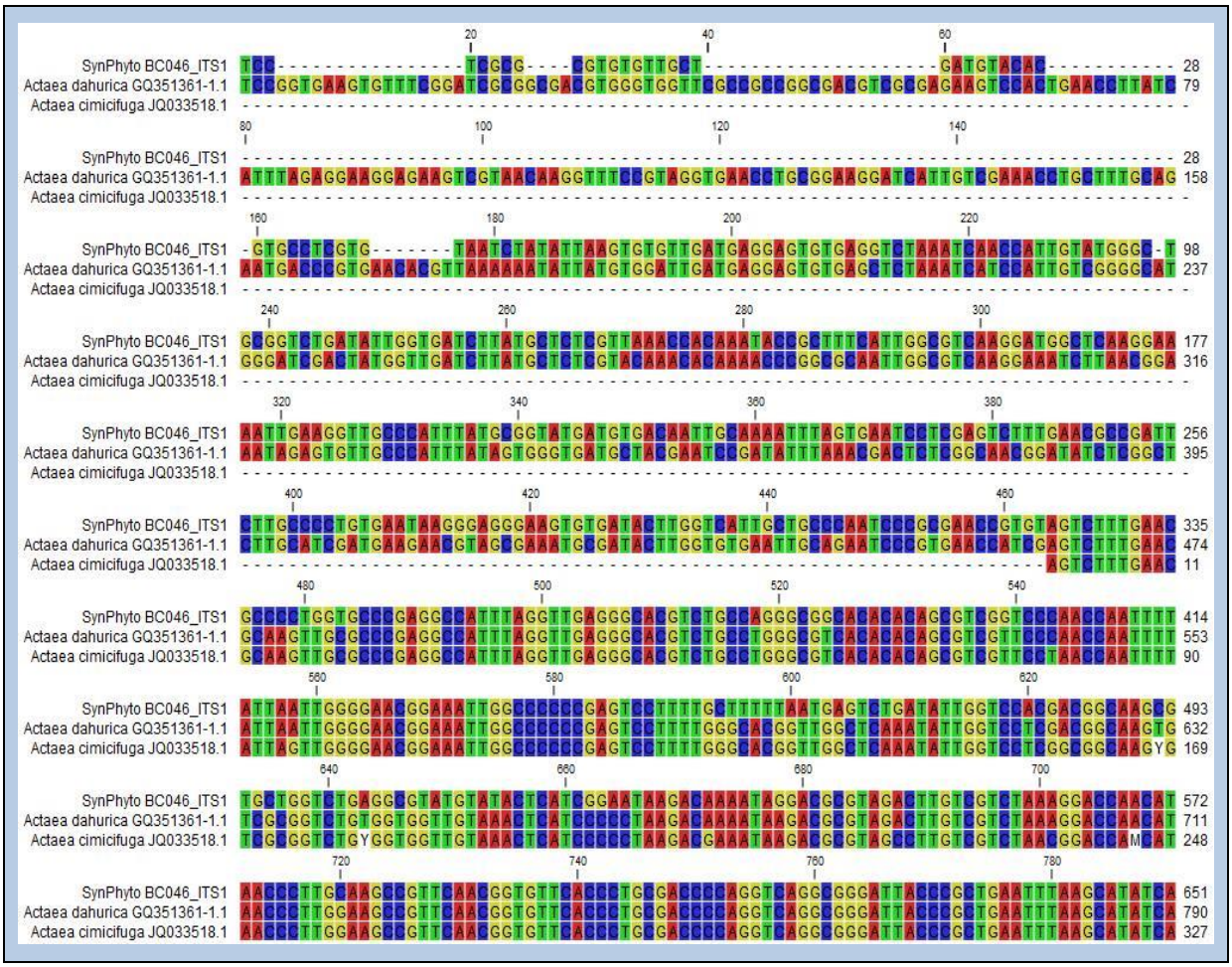


Figure 26: Alignments of *A. cimicifuga* material from SynoPhyto with *A. dahurica* and *A. cimicifuga* sequences available from GenBank.

The *A. racemosa* material from Staffort, DHU, was already positively identified using morphological means. It also was clearly sequenced and assigned identity as *A. racemosa.*

2.4.1.1.1 Assessment of vouchered DNA samples from Botanical garden DNA banks

For the MSc by Research degree project (Outlined in Chapter 3. Further development of the PlantID assay) (Williams 2012), the analysis of DNA sample from Kew, Royal Botanical Gardens, i.e. identifying the sequences using BLAST, was not carried out to the same extent. The DNA samples were sequenced but the data was checked for quality rather than identification. At the time it was taken for granted that the samples were identified correctly. These samples were used to develop species specific primer pairs with differing sized products from one species to another to enable identification within a mixed sample.

Table 17: A list of relevant information of DNA samples used to develop the plantID assay

Species	Source	DNA bank ID	Sequence agreement?	Successful in final MSc PlantID assay?
Actaea racemosa	Kew	24092	Yes	Yes
Actaea cordifolia	Kew	20113	Yes	No
Actaea pachypoda	Kew	550	Yes	No
Actaea dahurica	Kew	-	No – *A. simplex*	No
Actaea cimicifuga	Kew	10294	Yes	Yes
Actaea heracleifolia	CAMAG	52785BRM	Sequence data was poor – closest match – *A. dahurica*	No
Actaea rubra	CAMAG	52544	Yes	No
Actaea podocarpa	CAMAG	53504BRM	Yes	Yes
Actaea simplex	Beeches Nursery	-	Yes	No
Caulophyllum thalictroides	Kew	28066	Sequence data was for fungal contamination	No

The *Actaea dahurica* DNA purchased from Kew was extracted from a vouchered sample that would have been identified morphologically from a taxonomist. The sequencing data however has a top hit for *Actaea simplex* so this will be explored further.

The alignments for the top hit, *Actaea simplex* and the top hit for *Actaea dahurica* were both studied. *Actaea simplex* has an ID of 99% and *Actaea dahurica* has an ID of 96%, both have coverage of 82%.

From looking at the alignments it seems that *Actaea simplex* is a match in this case. For the purpose of developing species specific primers, this DNA sample would not be appropriate.

It was however at the time used for developing *A. dahurica* species specific primers for the PlantID assay (described in Chapter 3. Further development of the PlantID assay). The designed *A. dahurica* primers were not successful and neither were the *A. simplex* species specific primers. Each primer set produced PCR products with a panel of other *Actaea* species. The obvious reason for this is that the wrong template was used for the *A. dahurica* species specific primer development which in turn prevented both sets of species primers from being specific at the time. This was revisited later on in the qPCR assay development as it was very desirable to be able to identify *A. dahurica* species (Chapter 4 Development of a qPCR assay for the authentication of *A. racemosa* and detection of potential adulterants). A new template was procured for the assay development but this could still not be included in PlantID due to the high melting temperature required for specificity. The plantID assay melting temperature had to be 62.7°C for specificity of certain primer pairs but could not be more as other primer pairs would not work at higher temperatures. The *A. heracleifolia* template used at the time gave a poor sequence trace also and the primers were not successfully optimised or included in any future assays.

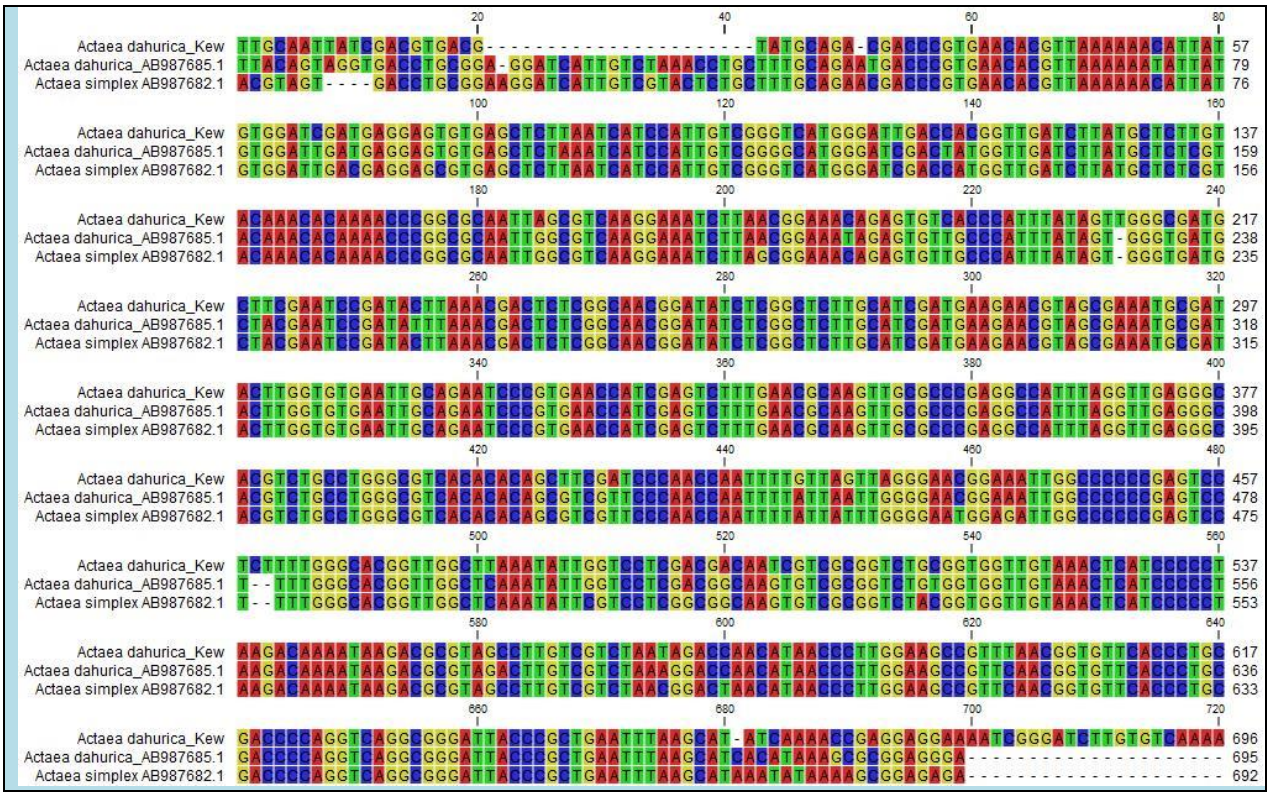


Figure 27: Comparison of alignments of potential matches of *Actaea dahurica* DNA from Kew - *Actaea simplex* and *Actaea dahurica*.

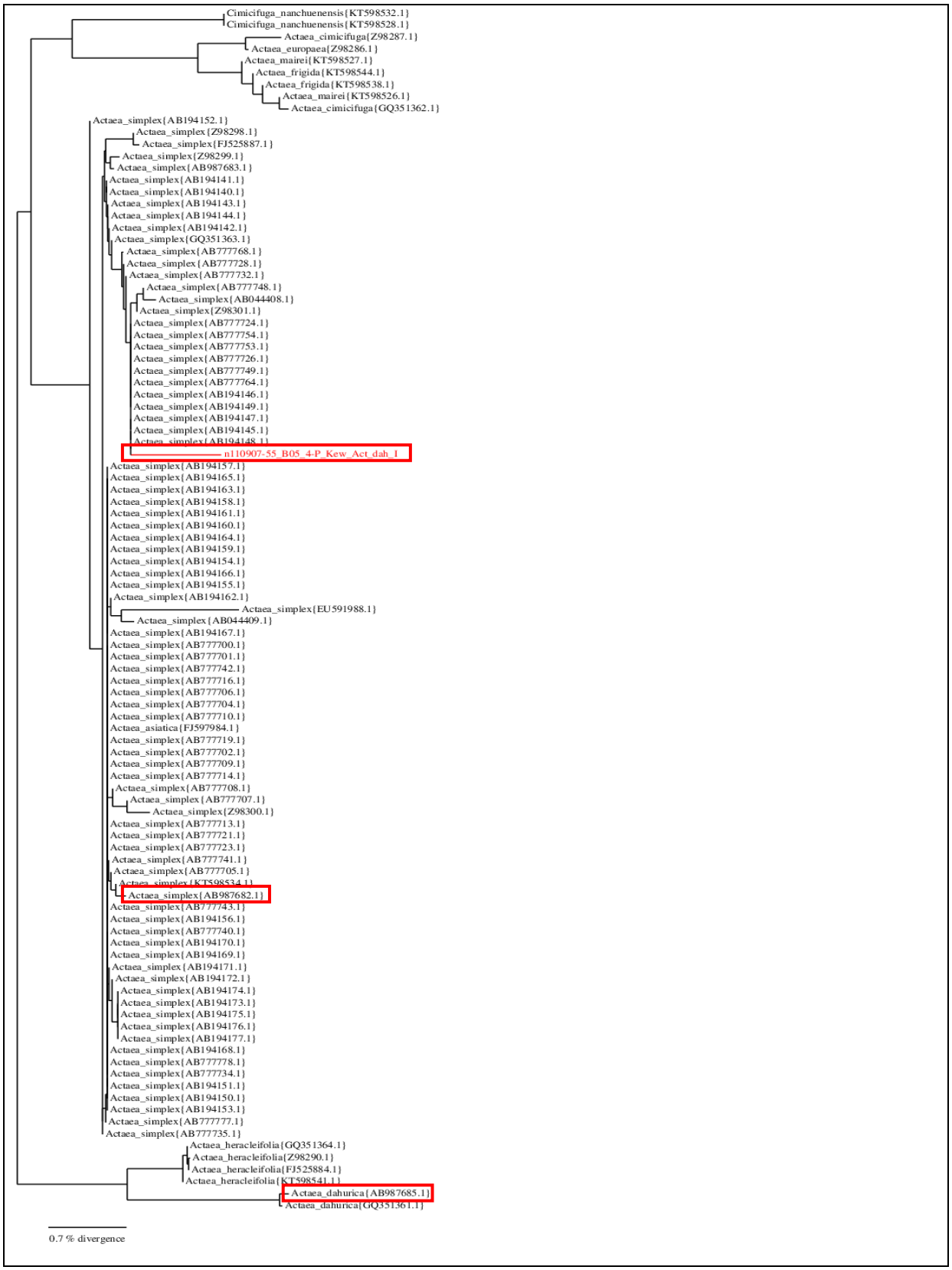


Figure 28: A tree diagram of the top 100 hits from BLAST when using the Kew DNA sample labelled *A. dahurica* as the query sequence.

In the end only three species were able to be included in the PlantID assay due to issues with some of the templates used. The same species specific primers would be used for development of qPCR assays and so further work will need to be carried out.

The species specific primers were first designed in 2009. At the time there were limited sequence entries in GenBank and the species specific primers were designed with these available sequences. In 2013 the qPCR assays began development. By 2015 there was much more sequence data for *Actaea* species deposited into GenBank which allowed the checking of the specificity of the primers on a larger pool of information and for sequenced samples to be identified. This helped tremendously with identifying and rectifying previous issues found.

3 Further Development of the PlantID Assay

3.1 Introduction

Adulteration of Black Cohosh with Asian Actaea species is a known occurrence (Jordan et al., 2010, Jiang et al., 2006, Jiang et al., 2011). In Chinese and Japanese medicine several Asian Actaea species are used in a medicine called Cimicifugae Rhizoma, in Chinese medicine this is called Sheng Ma. This is also the Latin name for Black Cohosh and therefore the medicine is sometimes marketed with the incorrect name. This can cause confusion and allow the wrong species to be used.

Due to the wild harvesting of Black Cohosh there is also the potential for contamination with other Native American species that grown in a similar habitat. These plants can also look very similar and could be mistaken for Black Cohosh and therefore collected by accident. Blue Cohosh, *Caulophyllum thalictroides* can also be labelled as Black Cohosh by mistake due to the similar common names.

Owing to the fact there are several potential adulterants of Black Cohosh it could be beneficial to have a test that is capable of detecting multiple adulterant species simultaneously.

The idea for PlantID came from Tobe et al 2008 who developed a one tube assay to detect 18 different mammalian species. The reaction was carried out in multiplex in one tube with a primer mix designed to produce different sized products for each species and then analysed using capillary electrophoresis (Tobe and Linacre, 2008).

This was also applied to the medicinal herb St John's Wort successfully. This was a good development of the original assay idea due to the similarity of the Hypericum species compared to the original work with very different species that were easy to distinguish. The Hypericum based assay was capable of distinguishing the authentic species *Hypericum perforatum* from three other Hypericum species; *Hypericum androsaemum, Hypericum athoum* and *Hypericum ascyron* (Howard et al., 2012).

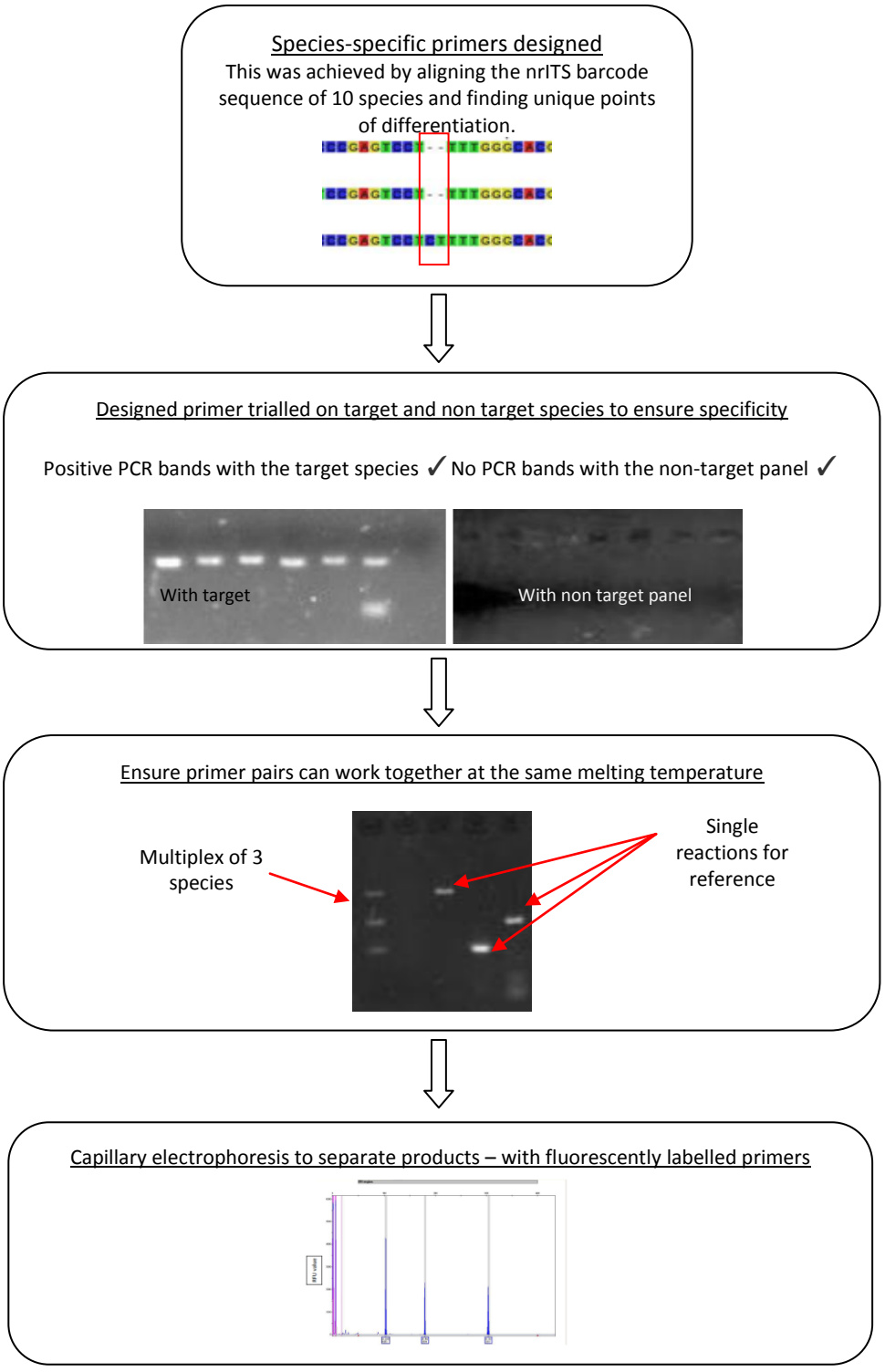


Figure 29: A flow chart to show the development of the PlantID assay for the differentiation of three *Actaea* species – primer design, primer optimisation, multiplex PCR optimisation and visualising separation of PCR products with capillary electrophoresis using fluorescently labelled primers.

In the MSc by Research degree completed in 2012 (Williams 2012), the PlantID assay was first developed for Black Cohosh. It was successful and capable of distinguishing between 3 different species of *Actaea*, namely *Actaea racemosa*, *Actaea cimicifuga* and *Actaea podocarpa*. Figure 29 displays the steps involved to develop the PlantID assay for *Actaea.*

The aim in the current piece of work is to increase the number of species that can be detected. There was issues with some of the primer pairs in the original work that could have been resolved if there had been more time available. In this piece of work the number of species included has been increased to five. This now includes Blue Cohosh, *Caulophyllum thalictroides* and *Actaea cordifolia.* The method has also been simplified from capillary electrophoresis to agarose gel electrophoresis.

At current there are no methods for detecting multiple species in Black Cohosh material to the same level as this assay.

The main outcomes of this chapter are to:

- Increase the number of detectable species to include *Caulophyllum thalictroides* and *Actaea cordifolia*. This will add to the current assay which is able to detect *Actaea racemosa, Actaea cimicifuga* and *Actaea podocarpa.*
- Trial other platforms for analysis including qPCR with high resolution melting (HRM). This method has been shown to be useful in identification of species in herbal medicine (Costa et al., 2016).

3.2 Materials and Method

3.2.1 Sourcing of plant materials and vouchered DNA samples

Vouchered DNA specimens were the favoured sample type to be used in the development of this assay, but supply was very limited and not available for all the species that were to be investigated. Although vouchered DNA specimens are identified by expert taxonomists, they were still further identified using sequencing as it was found in the previous chapter that even these experts can assign incorrect identity. The following samples were used; from the Royal Botanic Gardens, Kew there were six vouchered DNA samples; *Actaea racemosa, Actaea pachypoda, Actaea dahurica, Actaea cimicifuga, Actaea cordifolia* and *Caulophyllum thalictroides*. Three of the samples were a gift from Dr Eike Reich (CAMAG Muttenz, Switzerland): *Actaea rubra, Actaea podocarpa* and *Actaea heracleifolia*. The remaining sample, *Actaea simplex,* was from a plant purchased from a commercial nursery (Beeches Nursery, Saffron Waldon, UK).

Table 18: A list of relevant information of DNA samples used to develop the plantID assay.

Species	Source	DNA bank ID	Collector (Kew)
Actaea racemosa	Kew	24092	HerbariumKewenseCultivatedPlants s.n., TaxonomicNote: 2006 S.Landrein (K) det. Cimicifuga racemosa (L.) Nutt.
Actaea cordifolia	Kew	20113	Chase
Actaea pachypoda	Kew	550	Chase
Actaea dahurica	Kew	24397	TCMK82
Actaea cimicifuga	Kew	10294	Chase
Actaea heracleifolia	CAMAG	52785BRM	
Actaea rubra	CAMAG	52544	
Actaea podocarpa	CAMAG	53504BRM	
Actaea simplex	Beeches Nursery		
Caulophyllum thalictroides	Kew	28066	Chase

In the previous chapter, sequencing was attempted for each of the templates in Table 18. It was found that the *Actaea dahurica* extraction was in fact *Actaea simplex.* Some of the templates could

not be identified using sequencing but all of the species that were included in the final assay except from *Caulophyllum thalictroides* were identified successfully with sequencing.

3.2.2 Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit

The DNA extractions were carried out using the method outlined in 2.2.2 Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit (Qiagen Inc., CA).

3.2.3 DNA clean up using isopropanol method

DNA cleans up where required were carried out as outlined in 2.2.3 DNA clean up using isopropanol method.

3.2.4 Basic DNA clean using the Qiagen DNeasy Mini Kit

DNA purchased from Royal Botanical Gardens, Kew, was cleaned up using the method outlined in 2.2.4 Basic DNA clean using the Qiagen DNeasy Mini Kit (Qiagen Inc., CA).

3.2.5 Primer Design

The primers that were used for the development of the PlantID assay were designed by Professor Adrian Slater using the software program AlleleID® (Premier Biosoft., USA). This was achieved by aligning the nrITS sequence of all the species of interest and inputting this data into AlleleID®. The program then finds differences in the sequences and designs primers specific to each species.

Table 19: A summary of analysis of the original Genbank sequences used to design species specific primers.

Species	Accession number	Name of sequence	Length (bp)	Author(s)	MOTU result
Caulophyllum thalictroides	L77158.1	Caulophyllum thalictroides (L.) Michx. internal transcribed spacer 1 (ITS1) DNA	234	Lee,N.S., Sang,T., Crawford,D.J., Yeau,S.H. and Kim,S.-C.	N/A – non-Actaea
Actaea racemosa	Z98296.1	Cimicifuga racemosa 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	607	Culham,A.	*Actaea racemosa*
Actaea dahurica	Z98284.1	Cimicifuga dahurica 5.8S nuclear rRNA gene and ITS1 and ITS2	604	Culham,A	*Actaea dahurica*
Actaea cimicifuga	Z98287.1	Cimicifuga foetida 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	604	Culham,A	*Actaea cimicifuga*
Actaea podocarpa	Z98280.1	Cimicifuga americana 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	605	Culham,A	*Actaea podocarpa*
Actaea rubra	Z98278.1	Actaea rubra 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	607	Culham,A	*Actaea rubra*
Actaea cordifolia	Z98297.1	Cimicifuga rubifolia 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	603	Culham,A	*Actaea cordifolia*
Actaea heracleifolia	Z98289.1	Cimicifuga heracleifolia 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	603	Culham,A	*Actaea heracleifolia*
Actaea simplex	Z98298.1	Actaea simplex 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	604	Culham,A	*Actaea simplex*
Actaea pachypoda	Z98277.1	Actaea pachypoda 5.8S nuclear rRNA gene and ITS1 and ITS2 DNA	608	Culham,A	*Actaea pachypoda*

There was limited sequence data for *Caulophyllum thalictroides.* Only two accessions are available on GenBank which are two separate sections of the region. This means that using BLAST for matching any samples is not very reliable and so were used with caution.

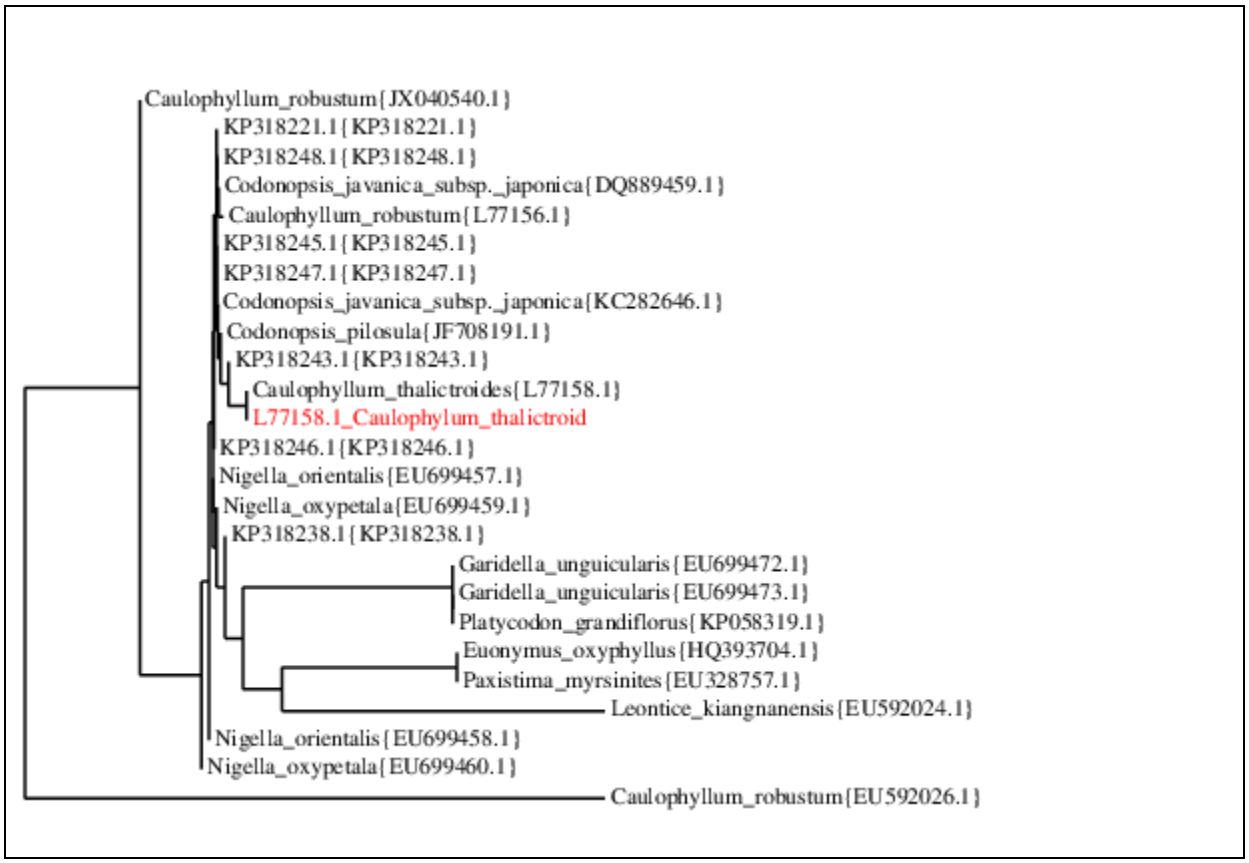


Figure 30: The results of using BLAST explorer when using a *Caulophyllum thalictroides* sequence as the query.

The primer pair for *Actaea cordifolia* was redesigned as the original design was not compatible in the PlantID assay. The primers were not suitable for the necessary melting temperature required for the assay. The new primer pairs were designed by studying the sequence alignment of the 9 *Actaea* species. They were based on the existing designed primers with extra base pairs added on to increase the annealing temperature. Several pairs were trialled and the most suitable pair was chosen.

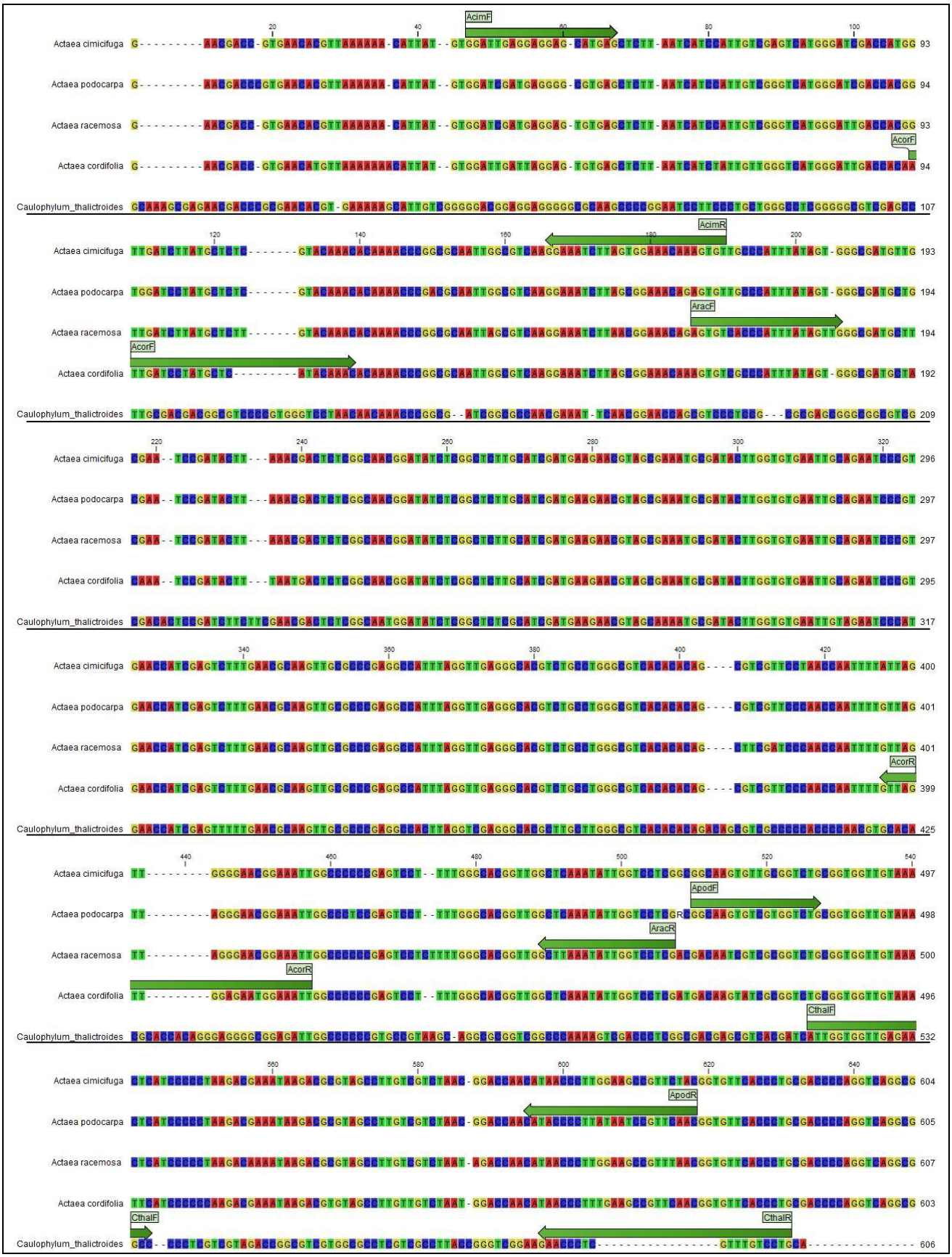


Figure 31: An alignment of the species included in PlantID – placement of included primers shown

Positioning of all primers on the nrITS sequence of each species can be found in the appendix.

3.2.6 Preparation of non-target panels of DNA

In order to test primer specificity, non-target panels were made up. This was achieved by creating mixtures of all the nrITS high fidelity reactions minus the species that the primers were designed for, i.e. Non-A.rac contained DNA for all species except *A. racemosa.* The protocol in Table 20 was followed to create all the non-target panels. For the non-target panels each ITS Hi-Fi reaction was added at 10 times the concentration of what was used for the target template. This is because by adding them together they become diluted 10 times. The target DNA templates and non-target panels remain a consistent concentration.

Table 20: A table to show which species were included in respective non-target DNA panels.

	DNA Templates									
Panel	***A.ram 4.9***	***A.rub 4.9***	***A.pac 4.9***	***A.her 3.9***	***A.pod 4.9***	***A.cor 4.9***	***A.dah 3.9***	***A.sim 4.9***	***A.cim 4.9***	***C.thal 4.9***
Non-A.rac	×	✓	✓	✓	✓	✓	✓	✓	✓	✓
Non-A.rub	✓	×	✓	✓	✓	✓	✓	✓	✓	✓
Non-A.pac	✓	✓	×	✓	✓	✓	✓	✓	✓	✓
Non-A.her	✓	✓	✓	×	✓	✓	✓	✓	✓	✓
Non-A.pod	✓	✓	✓	✓	×	✓	✓	✓	✓	✓
Non-A.cor	✓	✓	✓	✓	✓	×	✓	✓	✓	✓
Non-A.dah	✓	✓	✓	✓	✓	✓	×	✓	✓	✓
Non-A.sim	✓	✓	✓	✓	✓	✓	✓	×	✓	✓
Non-A.cim	✓	✓	✓	✓	✓	✓	✓	✓	×	✓
Non-C.thal	✓	✓	✓	✓	✓	✓	✓	✓	✓	×

3.2.7 Standard (End-point) Polymerase Chain Reaction

This is outlined in 2.2.5 Standard (End-point) Polymerase Chain Reaction.

3.2.8 Multiplex Polymerase Chain Reaction

The Qiagen Multiplex PCR kit (Qiagen Inc., CA) was used. The kit consisted of 2× Qiagen Multiplex PCR Master Mix and RNase free water. To start with, reactions were made up to 50 µL, and included 25µL of 2× Qiagen Multiplex PCR Master Mix, 5 µL of 10× primer mix (IDT, Iowa USA and Invitrogen, Carlsbad, CA, USA), 2 µL of template DNA and 18 µL of RNase free water. The reactions were made up in 0.2 mL tubes. After development in competency, the reactions were made up to 10 µL with all

components of the reaction in the same proportion as before. The primer mix was prepared to start with following suggested protocol, as by Table 21 below.

Table 21: Preparation of a 10 × primer mix

Components	Primer stock concentration	
	25 µM	100 µM
Primers (2 µM)	4 µl	1 µl
Water	Variable	Variable
Total volume	50 µl	50 µl

In order to balance efficient and inefficient reactions, the primer concentration was altered and optimised in the final mix.

The following conditions, Table 22, were used for amplification after optimisation;

Table 22: Conditions required for Multiplex PCR with the candidate primers selected for PlantID.

Initial Denature	30 cycles			Final Extension
	Denature	**Anneal**	**Extend**	
95°C	95°C	62.4°C	72°C	72°C
15 minutes	30 seconds	90 seconds	60 seconds	10 minutes

3.2.9 Gel Electrophoresis using agarose

PCR reactions were analysed using gel electrophoresis with the method outlined in 2.2.6 Gel Electrophoresis using agarose.

3.2.10 High resolution melting (HRM)

The MeltDoctor™ Master Mix (Applied Biosystems, California) was utilised for these experiments. Each reaction was made up as outlined in Table 23 and cycling parameters are outlined in Table 24.

Table 23: The recipe required for each high resolution melting reaction using MeltDoctor

Component	Amount (µL)
MeltDoctor™ MasterMix 2 X	5
Forward Primer	0.3
Reverse Primer	0.3
DNA Template	1
Nuclease free water	3.4

Each test sample was analysed in triplicate. Samples were evaluated using the StepOnePlus qPCR machine (Applied Biosystems, California) under the conditions outlined in Table 24. After cycling was complete, the experiment was opened with Applied Biosystems HRM software for analysis.

Table 24: Cycling conditions for reactions with MeltDoctor™

Initial Denature 1 cycle	40 cycles			Melt curve		
	Denature	**Anneal**	**Plate read**	**Start temperature**	**End temperature**	**Rate** (Plate read each incremental increase)
95°C	95°C	62.4°C		60°C	95°C	0.3°C/Min
10 minutes	15 seconds	1 minute				

3.3 Previous development in the MSc by Research project

IMPORTANT NOTE: All work in this section was carried out during the MSc by Research degree in 2012 by Sarah Williams and is only included in this thesis for background purposes (Williams 2012).

The main development covered in the MSc by Research project was to optimise the primers.

This involved testing pairs of species specific primers with target DNA to ensure that they were capable of amplifying the desired species. The next step was to test the species specific primers with non-target panels. If any amplification occurred then the temperature of the annealing stage of the PCR reaction was increased. If increasing the annealing temperature did not resolve the issue the primers were discarded. If the new annealing temperature was then too high to amplify the target

then the primer pair was again discarded. The requirement was for the species specific primer pair to amplify only the target at a reasonable temperature. The results of the testing are summarised below in Table 25.

Table 25: Results of optimisation for species specific primers

Target Species	Forward Primer	Reverse Primer	Original Tm (°C)	Optimised Tm (°C)	Candidate?	Number of Candidates
A. racemosa	C.ram F.1.5	C.ram R.1.4	65.3	-	Failed to amplify target	2
A. racemosa	C.ram F.1.4	C.ram R.1.4	66.7	-	No stringency	
A. racemosa	C.ram F.1.3	C.ram R.1.4	65.8	-	No stringency	
A. racemosa	C.ram F.1.2	C.ram R.1.3	56.4	**57**	Candidate	
A. racemosa	C.ram F.1.1	C.ram R.1.2	52.0	**60**	Optimised Tm too high	
A. racemosa	C.ram F.1.1	C.ram R.1.1	52.4	**57**	Candidate	
A. rubra	A.rub F.1.2	A.rub R.1.6	61	**65**	Optimised Tm too high	0
A. rubra	A.rub F.1.1	A.rub R.1.5	64.7	**68**	Optimised Tm too high	
A. rubra	A.rub F.1.2	A.rub R.1.4	62.1	**66**	Optimised Tm too high	
A. rubra	A.rub F.1.2	A.rub R.1.3	61.7	**65**	Optimised Tm too high	
A. rubra	A.rub F.1.1	A.rub R.1.2	64	**67**	Optimised Tm too high	
A. rubra	A.rub F.1.1	A.rub R.1.1	64.4	**68**	Optimised Tm too high	
A. pachypoda	A.pac F.1.6	A.pac R.1.2	67.8	-	No stringency	0
A. pachypoda	A.pac F.1.5	A.pac R.1.2	67.8	-	No stringency	
A. pachypoda	A.pac F.1.4	A.pac R.1.3	63.8	**68**	Optimised Tm too high	
A. pachypoda	A.pac F.1.3	A.pac R.1.2	67.8	-	No stringency	
A. pachypoda	A.pac F.1.2	A.pac R.1.2	67.1	-	No stringency	
A. pachypoda	A.pac F.1.1	A.pac R.1.1	57.0	61	Optimised Tm too high	
A. dahurica	A.dah F.1.2	A.dah R.1.5	62.7	**65**	Optimised Tm too high	0
A. dahurica	A.dah F.1.3	A.dah R.1.3	55.0	**57**	Optimised Tm too high	
A. dahurica	A.dah F.1.2	A.dah R.1.4	63.3	**66**	Optimised Tm too high	
A. dahurica	A.dah F.1.1	A.dah R.1.3	55.3	**57**	Optimised Tm too high	
A. dahurica	A.dah F.1.2	A.dah R.1.2	63.3	**65**	Optimised Tm too high	
A. dahurica	A.dah F.1.1	A.dah R.1.1	55.0	**58**	Optimised Tm too high	
A. heracleifolia	A.her F.1.3	A.her R.1.1	60.3	-	Failed to amplify target	0
A. heracleifolia	A.her F.1.2	A.her R.1.4	59.0	**63**	Optimised Tm too high	
A. heracleifolia	A.her F.1.3	A.her R.1.2	60.5	**66**	Optimised Tm too high	
A. heracleifolia	A.her F.1.2	A.her R.1.3	59.0	**64**	Optimised Tm too high	
A. heracleifolia	A.her F.1.1	A.her R.1.2	60.2	**65**	Optimised Tm too high	
A. heracleifolia	A.her F.1.1	A.her R.1.1	60.1	-	Failed to amplify target	
A. simplex	A.sim F.1.4	A.sim R.1.4	57.8	**64**	Optimised Tm too high	0
A. simplex	A.sim F.1.4	A.sim R.1.3	58.4	-	Failed to amplify target	
A. simplex	A.sim F.1.2	A.sim R.1.4	57.4	-	Failed to amplify target	
A. simplex	A.sim F.1.3	A.sim R.1.3	58.7	**64**	Optimised Tm too high	
A. simplex	A.sim F.1.2	A.sim R.1.2	57.3	**63**	Optimised Tm too high	
A. simplex	A.sim F.1.1	A.sim R.1.1	67.0	-	No stringency	
A. podocarpa	A.pod F.1.3	A.pod R.1.6	65.8	-	Failed to amplify target	1
A. podocarpa	A.pod F.1.1	A.pod R.1.5	61.2	-	Failed to amplify target	
A. podocarpa	A.pod F.1.3	A.pod R.1.4	66	-	Failed to amplify target	
A. podocarpa	A.pod F.1.2	A.pod R.1.3	65.6	-	No stringency	
A. podocarpa	A.pod F.1.1	A.pod R.1.2	61.9	**62.4**	Candidate	
A. podocarpa	A.pod F.1.1	A.pod R.1.1	63.2	**68**	Optimised Tm too high	
A. cimicifuga	A.cim F.1.5	A.cim R.1.5	63.4	**63.4**	Candidate	5
A. cimicifuga	A.cim F.1.4	A.cim R.1.4	61.6	**61.6**	Candidate	
A. cimicifuga	A.cim F.1.3	A.cim R.1.3	64.5	**67**	Optimised Tm to high	
A. cimicifuga	A.cim F.1.1	A.cim R.1.2	59.5	**59.5**	Candidate	
A. cimicifuga	A.cim F.1.2	A.cim R.1.1	59.5	**60**	Candidate	
A. cimicifuga	A.cim F.1.1	A.cim R.1.1	60	**63**	Candidate	
A. cordifolia	A.cor F.1.6	A.cor R.1.3	62.2	**63**	Optimised Tm too high	5

A. cordifolia	A.cor F.1.5	A.cor R.1.1	52.9	-	Candidate	
A. cordifolia	A.cor F.1.4	A.cor R.1.2	63.8	**65**	Candidate	
A. cordifolia	A.cor F.1.3	A.cor R.1.1	52.9	-	Candidate	
A. cordifolia	A.cor F.1.2	A.cor R.1.1	52.9	**54**	Candidate	
A. cordifolia	A.cor F.1.1	A.cor R.1.1	52.9	**54**	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	57.3	-	Failed to amplify target	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	65.3	-	Failed to amplify target	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	66.5	-	Failed to amplify target	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	62.9	-	Failed to amplify target	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**64.4**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**63**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**59.8**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**59.8**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**57.9**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**57.9**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**57.3**	-	Candidate	
C. thalictroides	C.thal F.1.1	C.thal R.1.1	**57.3**	-	Candidate	

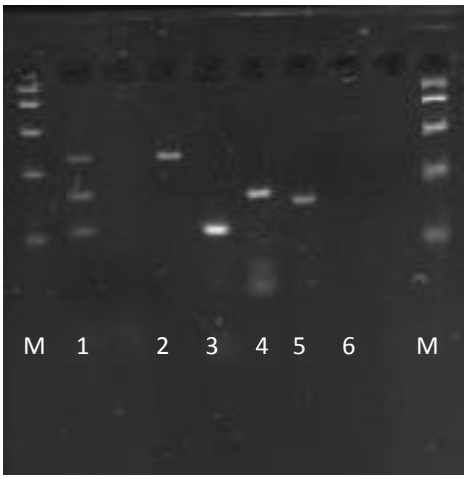
The next step was to choose primers to take on to the next stage of multiplex optimisation. Table 26 shows which primers were chosen.

Table 26: The primer pairs selected for multiplex optimisation.

Species	Sense Primer	Sequence	Antisense Primer	Sequence	Size of product (bp)	Tm (°C)
Actaea racemosa	Cram.F.1.2		Cram.R.1.1		304	57
Actaea podocarpa	Came.F.1.1		Came.R.1.2		108	62.4
Actaea cimicifuga	Cfoe.F.1.4		Cfoe.R.1.4		180	59.5
Actaea cordifolia	Crub.F.1.5		Crub.R.1.1		312	52.9
Caulophyllum thalictroides	Ctha.F.1.5		Ctha.R.1.4		123	57.3

The next step was to check if the primers would interact once used in the same reaction. Primer dimer software was used to this end and showed no issues.

A new target panel of all five species was made up. A primer mix was also made up. The multiplex reaction was trialled with the five species. Singleplex reactions were also made up.



1) Multiplex using a primer mix with all 5 primer combinations and a DNA mix with all 5 templates.

2) *A. racemosa* singleplex
3) *A. podocarpa* singleplex
4) *A. foetida* singleplex
5) *C. thalictroides* singleplex
6) *A. cordifolia* singleplex

M) Molecular markers – 2000bp, 1000bp, 500bp, 250bp and 100bp.

Figure 32: Multiplex and singleplex reactions for PlantID candidate species.

The annealing temperature required of the multiplex reaction was too high for *A. cordifolia* specific primers to produce a product. They had to be removed from the multiplex at this stage. There were also problems with *C. thalictroides* and this also had to be removed. The fluorescently labelled primers ordered for fragment analysis did not produce a product. In the end the PlantID assay was capable of detecting and discriminating between 3 species as shown below in Figure 33.

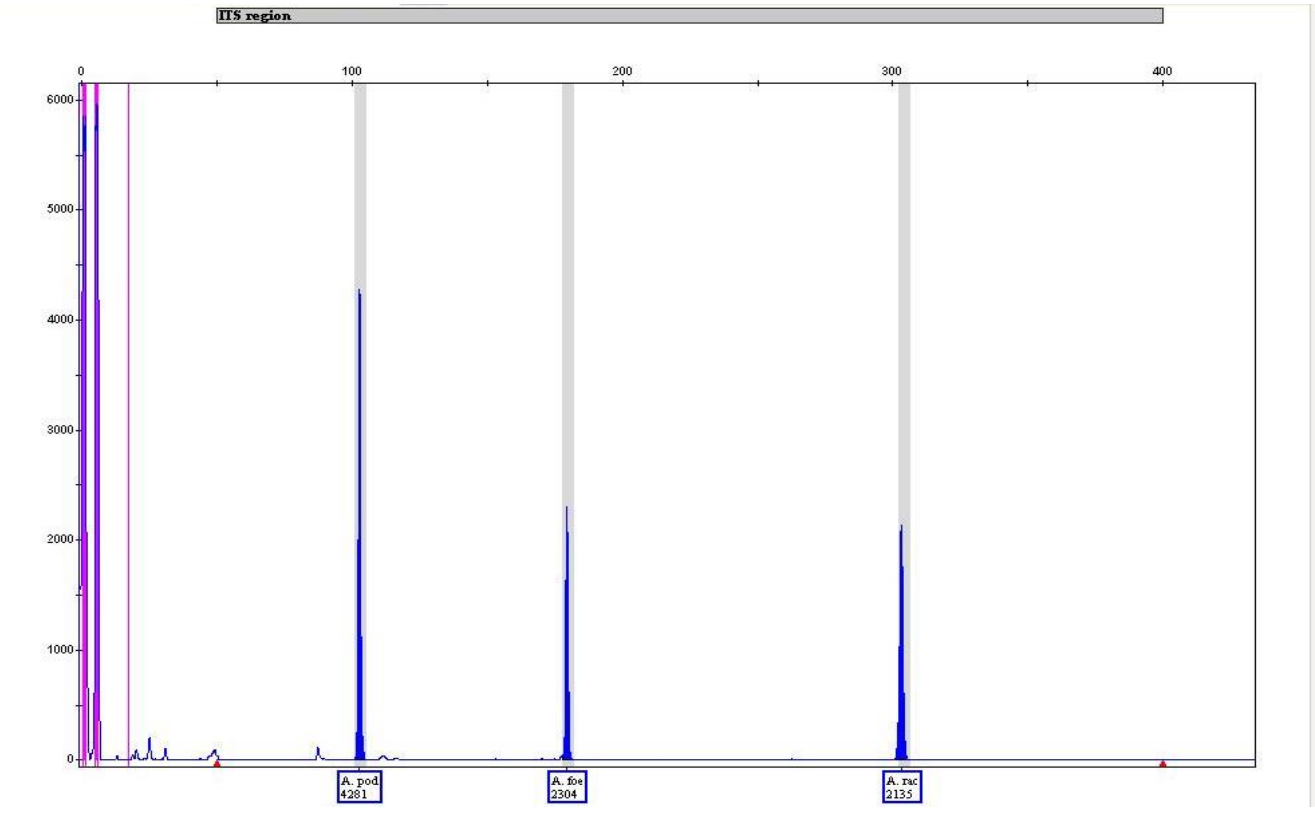


Figure 33: Fragment analysis results of the Black Cohosh PlantID assay.

Due to time constraints of the MSc by Research project the PlantID assay was only developed to this point.

For the PhD project the aim was to optimise another *C. thalictroides* primer pair and also to redesign an *A. cordifolia* primer to work at the required multiplex temperature. This would bring the overall number of distinguishable species from 3 to 5 species. Other platforms of analysis would also be explored.

3.4 Results and discussion

As mentioned, the aim was to increase the number of distinguishable species from 3, as seen in the MSc work to 5 species. The evaluation of the MSc work showed that it would be possible to detect *A. cordifolia* and *C. thalictroides* with some further work. The reason that *A. cordifolia* was not an option for the original assay is that the melting temperature of the main assay was too high. The working temperature for the assay had to be so in order for *A. cimicifuga* primers to be specific. Anything lower and non-specific amplification would occur. There was another primer pair option for *A. cordifolia* but the melting temperature was 65°C and none of the *A. racemosa* primers would work at this temperature. The solution to this issue was to redesign the *A. cordifolia* specific primers with the outcome of increasing the melting temperature. This could be achieved by increasing the length of the primers. Four primers were designed yielding 8 possible combinations. These primers were trialled in the same way as the previous primers and an annealing temperature of 62.7°C was used. They were first used to amplify the target DNA – *A. cordifolia*. Of the 8 combinations, 4 were able to produce a product. The next stage was to use the successful primers in respective reactions with the non-target panel. Again all 4 combinations did not amplify. One of these pairs was put forward for testing within the multiplex reaction.

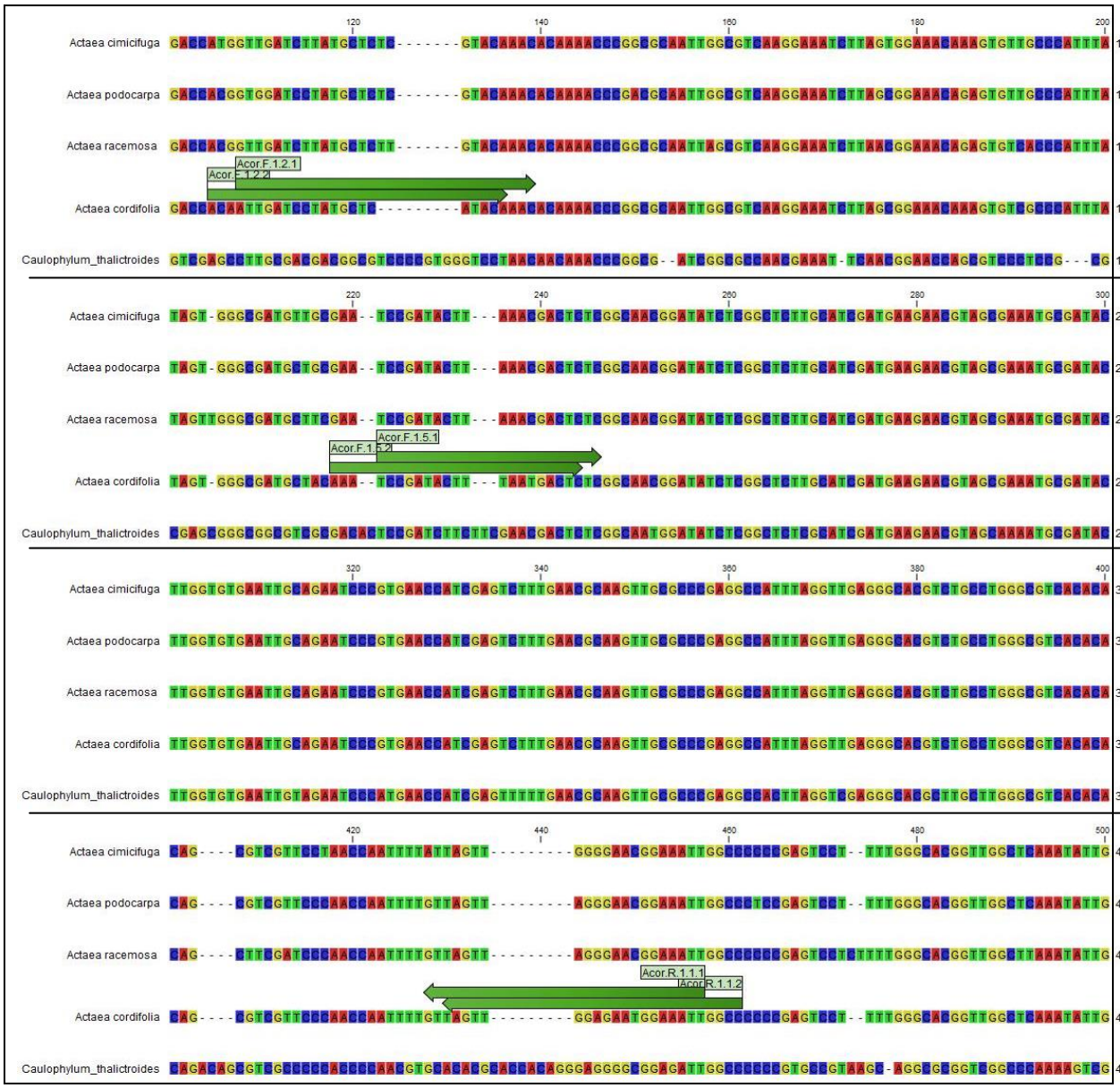
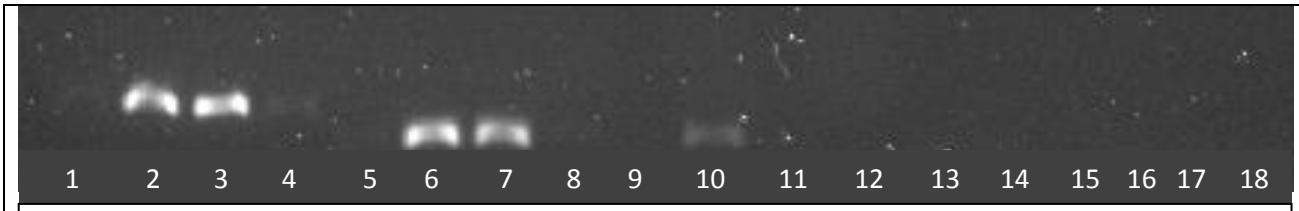


Figure 34: An alignment of PlantID species and placement of newly designed *A. cordifolia* specific primers



With *A. cordifolia* template		With non-*A. cordifolia* panel	
1 – F.1.2.1 + R.1.1.1	*6 – F.1.5.2 + R.1.1.2*	11 – F.1.2.1 + R.1.1.1	16 – F.1.5.2 + R.1.1.2
2 – F.1.2.2 + R.1.1.2	*7 – F.1.5.1 + R.1.1.2*	12 – F.1.2.2 + R.1.1.2	17 – F.1.5.1 + R.1.1.2
3 – F.1.2.1 + R.1.1.2	8 – F.1.5.2 + R.1.1.1	13 – F.1.2.1 + R.1.1.2	18 – F.1.5.2 + R.1.1.1
4 – F.1.2.2 + R.1.1.1	9 – No template	14 – F.1.2.2 + R.1.1.1	
5 – F.1.5.1 + R.1.1.1	10 – Positive control	15 – F.1.5.1 + R.1.1.1	

Figure 35: Testing of newly designed *A. cordifolia* primers

For the *C. thalictroides* specific primers it was unknown why the primers ceased to work and due to time constraints during the MSc degree this was not explored further. Upon closer analysis it would appear that the original primers selected did not yield a good product and the presence of an artefact on the gel obscured this fact. Therefore it was a simple case of selecting and trialling another potential primer pair.

Primer Pair	Sense primer	Anti-sense primer	Tm (°C)
1	C.tha F.1.5	C.tha R.1.4	57.3
2	C.tha F.1.4	C.tha R.1.3	65.5
3	C.tha F.1.3	C.tha R.1.3	66.8
4	C.tha F.1.2	C.tha R.1.1	63.0
5	C.tha F.1.2	C.tha R.1.2	64.4
6	C.tha F.1.1	C.tha R.1.1	63.0
7	C.tha F.2.6	C.tha R.2.2	59.8
8	C.tha F.2.5	C.tha R.2.2	59.8
9	C.tha F.2.4	C.tha R.2.1	57.9
10	C.tha F.2.3	C.tha R.2.1	57.9
11	C.tha F.2.2	C.tha R.2.1	57.3
12	C.tha F.2.1	C.tha R.2.1	57.3
13	NTC		

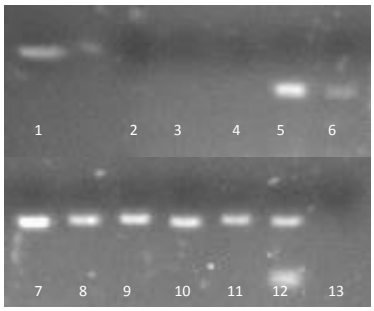


Figure 36: The previous results for *C. thalictroides* primer pair trials (Williams, 2012). Positive PCR amplification shown by the presence of a band on the accompanying agarose gel.

Table 27: The concentration of each primer that was included in the PlantID assay for the first set of experiments

Specific species template	Primer Names	Concentration (nM)	Size of product (bp)
A. racemosa	Arac F.1.2 and Arac R.1.3	400	304
A. podocarpa	Apod F.1.1 and Apod R.1.2	200	108
A. cimicifuga	Acim F.1.4 and Acim R.1.4	300	180
A. cordifolia	Acor F.1.2.2 and Acor R.1.1.2	200	322
C. thalictroides	Cthal F.2.6 and Cthal R.2.2	400	87

These primers were designed in 2009 and at the time there was a limited amount of sequence data available from GenBank to use. Since this time there has been much more data added to the database. It was sensible to check through the available sequences in Genbank again to see how specific the primers are. Each set of primers was checked using BLAST (Basic Local Alignment Search

Tool). Only results with coverage of 100% were considered matches due to the search being for the primers and the entire primer must match the sequences to be considered.

Table 28: Results of specific primer BLAST searches.

Species	Primer	Match(es)	Coverage
Actaea racemosa	Aram.F.1.2	***Actaea racemosa***	100%
Actaea racemosa	Aram.R.1.1	***Actaea racemosa***	100%
		Actaea heracleifolia	100%
		Ranunculus haastii subsp. piliferus	100%
Actaea podocarpa	Apod.F.1.1	***Actaea podocarpa***	100%
Actaea podocarpa	Apod.R.1.1	***Actaea podocarpa***	100%
Actaea cordifolia	Acor.F.1.5.1	***Actaea cordifolia***	100%
		Actaea elata	100%
Actaea cordifolia	Acor.R.1.1.1	***Actaea cordifolia***	100%
		Actaea elata	100%
		Actaea arizonica	100%
Caulophyllum thalictroides	Ctha.F.1.5	***Caulophyllum thalictroides***	100%
		Neurada procumbens	100%
		Caulophyllum robustum	100%
Caulophyllum thalictroides	Ctha.R.1.4	***Caulophyllum thalictroides***	100%
		Caulophyllum robustum	100%
Actaea cimicifuga	Acim.F.1.4	***Actaea cimicifuga***	100%
		Actaea brachycarpa	100%
		Actaea frigida	100%
		Actaea yunnanensis	100%
		Actaea mairei	100%
		Actaea kashmiriana	100%
		Actaea europaea	100%
Actaea cimicifuga	Acim.R.1.4	***Actaea cimicifuga***	100%
		Actaea brachycarpa	100%
		Actaea frigida	100%
		Actaea yunnanensis	100%
		Actaea mairei	100%
		Actaea kashmiriana	100%
		Actaea europaea	100%

From the search it found that the *Actaea racemosa* forward primer was specific only to *Actaea racemosa.* The reverse primers matched to *Actaea racemosa* but also to *Actaea heracleifolia* and an unrelated species of Rannunculus. This was not an issue as the forward primer was completely specific ensuring that in combination the primer pair would be specific to *Actaea racemosa.* The *Actaea podocarpa* primers were both completely specific to *Actaea podocarpa.* The *Actaea cordifolia* primers were both a match to *Actaea cordifolia* but were also both a match to *Actaea elata. Actaea elata* is not a known adulterant for Black Cohosh so in this case is not a concern. The reverse primer also matched to *Actaea arizonica* but as the forward primer was not a match this would not cause

any issues. The *Caulophyllum thalictroides* primers were a match to *Caulophyllum thalictroides* but also were both a match to *Caulophyllum robustrum.* In the previous chapter it was shown that there is only one sequence entry to *Caulophyllum thalictroides.* This again is not a known adulterant of Black Cohosh and so was not a concern. The forward primer was a match to *Neurada procumbens* but the reverse was not. The *Actaea cimicifuga* primers were both a match for various other Asian *Actaea* species not included in the project as they are not known adulterants of Black Cohosh.

There was also limited data from the Asian Actaea species. The sequences were organised into a tree to assess how similar they are and if the sequences of the same names species are grouped together. *Actaea elata* appears to be reasonably differentiated from the other included species but the rest are mixed up with the *Actaea cimicifuga* branch. This raises questions about the identification of these species. They could potentially be *Actaea cimicifuga.* There was limited numbers of sequence data for these species with many only having one accession. As these species are not known adulterants of Black Cohosh also, this was not of concern.

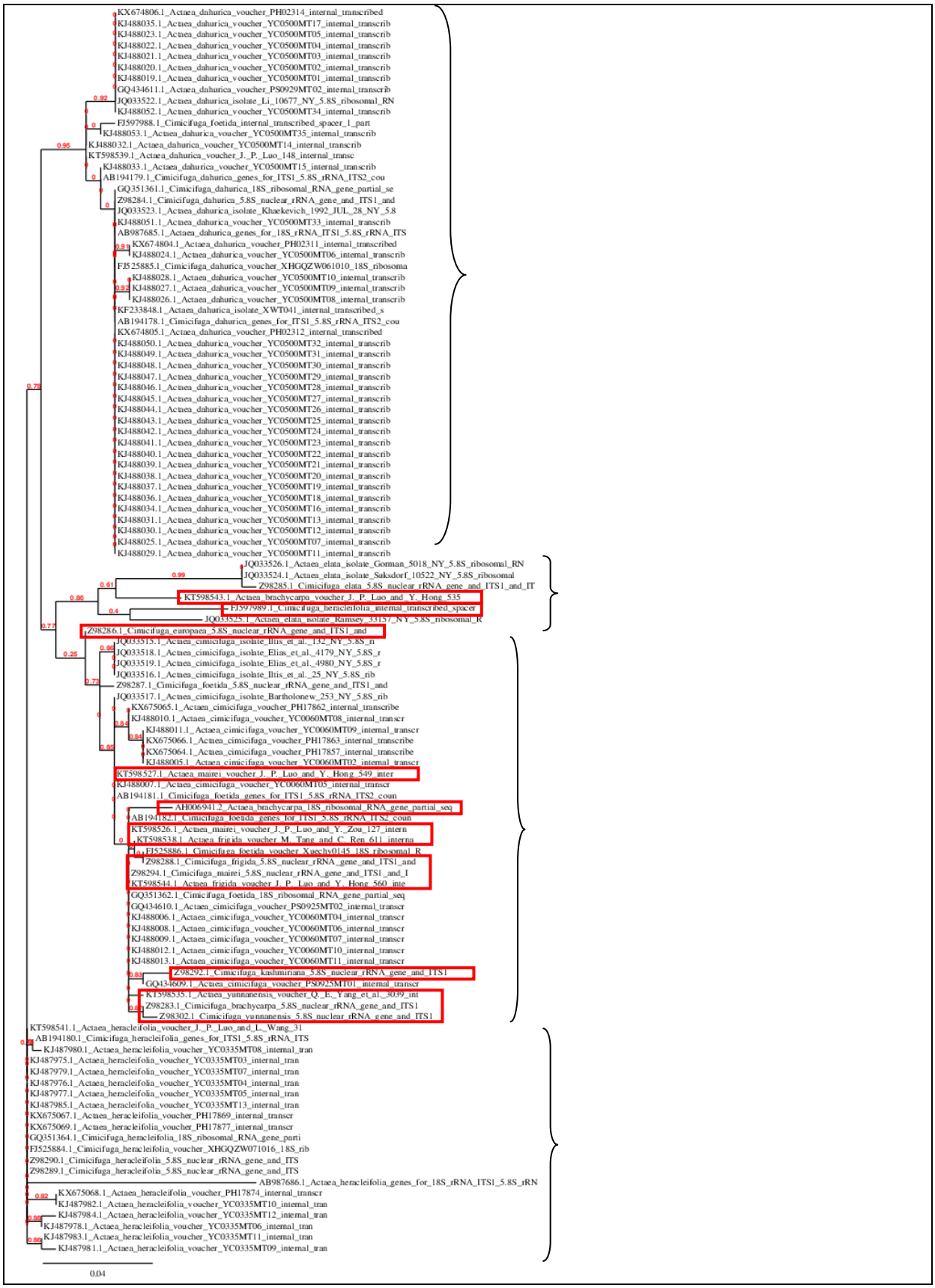
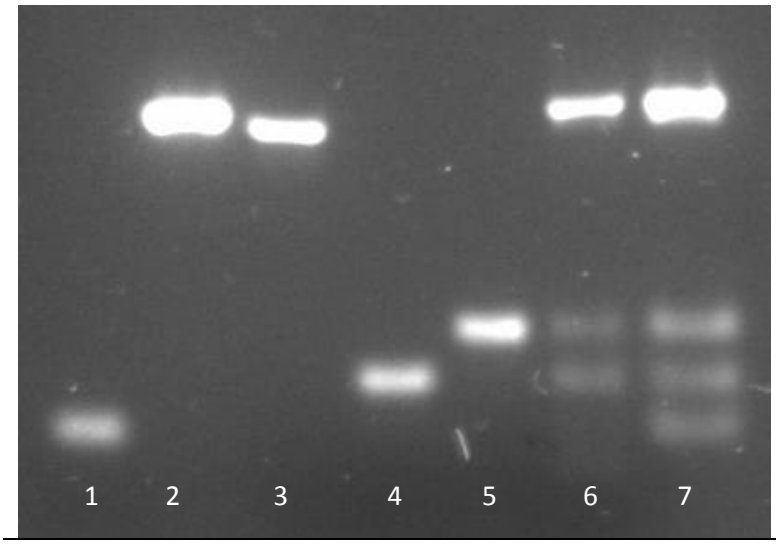


Figure 37: A tree diagram of Asian *Actaea* species to show similarity of sequences



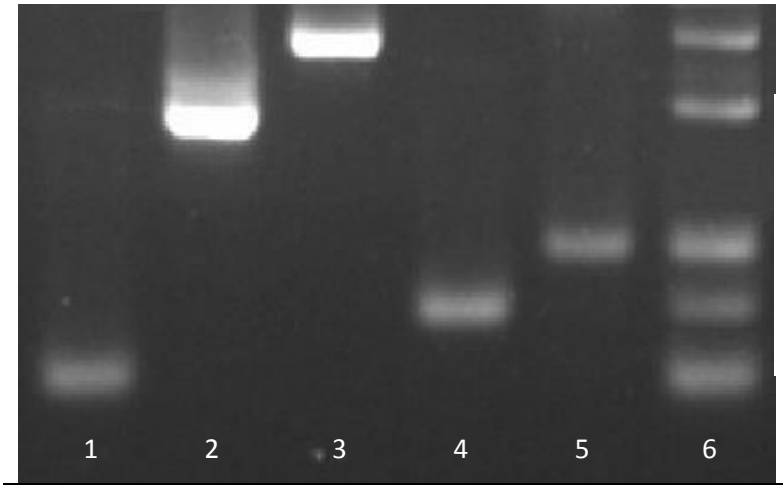
1 – *C. thalictroides*
2 – *A. cordifolia*
3 – *A. racemosa*
4 – *A. podocarpa*
5 – *A. cimicifuga*
6 – Multiplex reaction with all templates
7 – (1-5) mix

Figure 38: A 3% agarose gel showing Singleplex reactions for each species (lanes 1-5), a multiplex reaction of the five included species (Lane 6) and a mixture of each of the singleplex reactions to use as a reference (Lane 7).

Each of the five templates was amplified in a singleplex reaction with the species specific-primers. The templates were also mixed together to be amplified in a multiplex reaction. These samples were then run on a 3% gel. There were thought to be issues with separation of the *A. cordifolia* (Lane 4) and *C. thalictroides* (Lane 1) product as only one band was showing in the multiplex reaction (Lane 6). To check this, each of the Singleplex reactions were mixed together (Lane 7) and run alongside the multiplex reaction. This revealed that the *C. thalictroides* product was not forming in the multiplex reaction despite forming a product in Singleplex. The concentration of this primer pair was increased. The other issue was that it appeared that the product size for *A. cordifolia* and *A. racemosa* were too similar to be separated using gel electrophoresis. Another primer pair was selected with a greater difference in size and used in the next experiment. A new primer mix was made as shown in Table 29.

Table 29: The concentrations of primers used in the primer mix for PlantID

Specific species template	Primer Names	Concentration (nM)	Size of product (bp)
A. racemosa	Arac F.1.2 and Arac R.1.3	500	304
A. podocarpa	Apod F.1.1 and Apod R.1.2	200	108
A. cimicifuga	Acim F.1.4 and Acim R.1.4	300	180
A. cordifolia	Acor F.1.5.1 and Acor R.1.1.2	100	223
C. thalictroides	Cthal F.2.6 and Cthal R.2.2	700	87



1 – *C. thalictroides*
2 – *A. cordifolia*
3 – *A. racemosa*
4 – *A. podocarpa*
5 – *A. cimicifuga*
6 – Multiplex reaction with all templates

Figure 39: A 3% agarose gel showing singleplex reactions of each of the included five species and a multiplex reaction of the five species combined

This next experiment worked very well. The gel was allowed to run for a longer time to allow good separation of the multiplex products. All of the products were present in the multiplex reaction and the new primer pair for *A. cordifolia* produced a smaller product which could be separated from the *A. racemosa* product. The increased concentration of *C. thalictroides* primers gave a product in the multiplex and so solved that issue.

The multiplex reaction was also carried out using qPCR and melt curve analysis. The reactions were monitored using Meltdoctor as a fluorescent signal. All amplification will be picked up the same and there will be no difference seen at the amplification stage. Standard melt curve analysis is not a strong enough technique to differentiate between the close sizes of some of the products produced by the primers. This is where high resolution melting (HRM) will be trialled to see if this can be overcome. During the MSc degree this method was not available. Although high resolution melting offers the ability to resolve very small changes in product size, other factors can make a big difference to the overall result including overall product size and GC content. Unfortunately one of the PCR products exceeds the maximum size of this technique. The maximum size is 250 base pairs and one of the products exceeded 300 base pairs. A test run was carried out regardless.

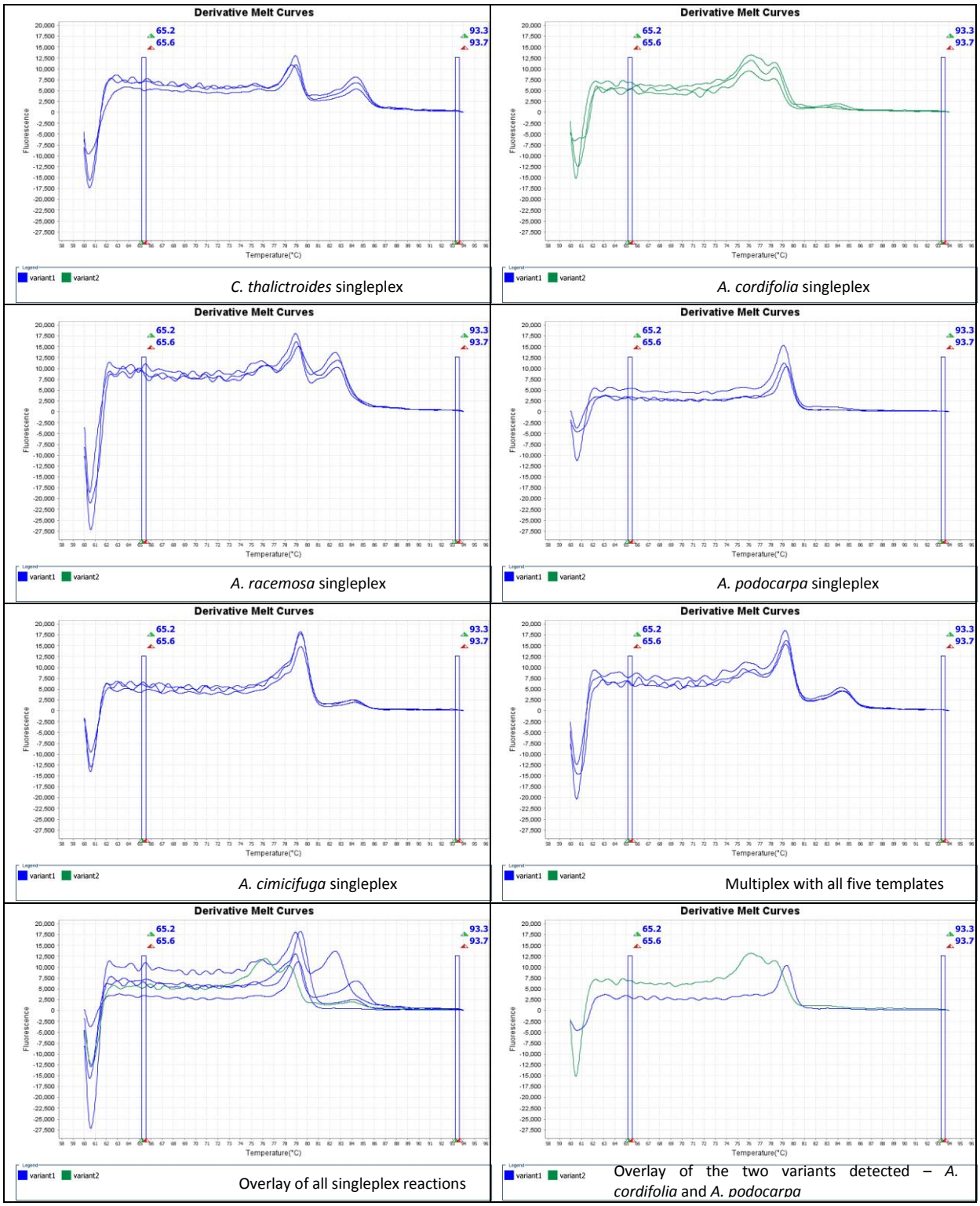


Figure 40: High resolution melting of singleplex and multiplex reactions – two variants shown – green variant shows *A. cordifolia* and the blue variant is the remaining species.

Unfortunately this was not very successful. The technique was only able to detect two variants. *Actaea cordifolia* was found as one variant and all the other species were detected as the same

variant. New primers would have to be designed for this platform and as the PCR with gel electrophoresis works fine no more time will be invested in this technique at present.

3.5 Conclusions and further work

The aim of this chapter was to further develop an assay capable of detecting mixtures of different plant species within one test. The importance and need for such a test is the ongoing difficulty of testing mixtures of polyherbal formulations. It also makes for an efficient test combing five species detection assays in one assay.

The plantID species detection capability was successfully increased from three to five. The more species that are added to the assay the more competition there is for the reagents required for amplification. This required more optimisation to balance the efficiencies of the primers included.

There are two levels to this assay; the species specific primers are designed to amplify only the species of interest and the primers are designed to amplify products of different sizes so that they are able to be combined into one reaction. It was sensible to amplify the templates in individual reactions so that it could be visualised in the multiplex reaction if the products were apparent and assess if the length of time for running the gel was sufficient.

The assay was originally visualised using capillary electrophoresis. This method gave a very firm conclusion for the results as the peaks were labelled with the species name if detected. The method is also more relatively expensive than other methods that exist. The capital cost of purchasing equipment is high and the running costs of such equipment are also high. It was possible in this work to visualise the multiplex on a simple agarose gel. This reduces the cost significantly in comparison to capillary electrophoresis and potentially saves time. Each reaction when using capillary electrophoresis takes around 20 minutes to complete. For the gel electrophoresis method the entire gel takes 2 hours to complete but there is the potential to run 80 samples simultaneously with the equipment used. Again this depends on the equipment available, as some high specification capillary

electrophoresis apparatus have 96 capillaries capable of running 96 reactions at once. The equipment available during the MSc had only one capillary.

To attempt to reduce the overall time of the assay, qPCR coupled with high resolution melting was trialled. This was not successful. If there was an interest to use this method in the future for this application, new primers would need to be designed specifically for HRM with products of less than 250 base pairs as the outcome. The method, like electrophoresis, would require the products to be of differing sizes enough to detect each. Other considerations are also the GC nucleotide content of the products as this affects the melting temperature. In the HRM assay performed in this work four products that were of differing size had the same melting temperature and so were indistinguishable.

The aim of this chapter was to extend the capability of the PlantID method to differentiate five species instead of three. This aim was achieved along with the aim to simplify the analysis of the multiplex PCR output using gel electrophoresis in place of capillary electrophoresis. The original method using Capillary electrophoresis is lengthy, troublesome and depends on the existence of expensive equipment and consumables. It was also found that the capillary electrophoresis method was not very reproducible between labs and between varying genetic analyser models. The outcome of this chapter was a much improved method with an extended capability of species detection.

The results of this chapter show the potential to develop assays for complex polyherbal formulations where sequencing and indeed most analytical chemistry based method would not be possible. This could be particularly useful in Traditional Chinese Medicine and Ayurvedic medicine where the products are made up of several different herbs. The plantID system can also be developed to test for multiple adulterants all in a one tube reaction which had not been a previous possibility.

4 Development of a qPCR assay for the authentication of *A. racemosa* and detection of potential adulterants

4.1 Introduction

Authentication of Black Cohosh has been a well-researched area with several research groups using barcoding grouped with UV spectroscopy, mass spectrometry and HPLC. There have also been DNA methods developed using restriction enzymes in various applications including RAPD, (Xu et al., 2002) RFLP, ARMS (Masada-Atsumi et al., 2014) and AFLP (Zerega et al., 2002). In this chapter novel qPCR based methods for detection of *Actaea racemosa, Actaea dahurica* and *Actaea cimicifuga* are presented as an easier and more rapid method compared to what is already available. The use of qPCR cuts out the need to run a separate diagnostic agarose gel as the reaction is plotted in real time and results are available immediately after the experiment is concluded. Techniques using qPCR have been developed for detection of plants containing aristolochic acid (Wu et al., 2015), for authentication of *Actaea cimicifuga* which is used in traditional Chinese medicine (Xue et al., 2009) and for authentication and detection of adulterants in the traditional herbal medicine *Drynaria fortunei* (Xue and Xue, 2008). The assay for *Actaea cimicifuga* (Xue et al., 2009) is based on melt curve analysis and includes detection of adulterant species; *Actaea heracleifolia, Actaea acerina* and *Actaea simplex*. These kinds of assays are not widely used at present and very little has been published. Mishra et al reviewed many available DNA based techniques for identification of herbal medicines in 2016 and mentions just two studies for qPCR methods (Mishra et al., 2016).

Lessons were learned in the last few years after the New York Attourney General Office issued cease and desist orders to three large shopping chains for several dietary supplements including some Black Cohosh brands. In the testing that was conducted they were not able to detect DNA of the

declared species in a large proportion of the products. It is well known within the field that is not always possible to amplify or sequence DNA barcode regions in products containing extracts due to the extraction process and potential heat and UV treatments of the raw materials. The testing that was carried out was from a single laboratory with no other identification techniques being employed. The scientist that was consulted also had no expert knowledge in the field of pharmacognosy, botany or natural product chemistry. The actions were deemed premature and without full scientific evidence. Chemical based methods should have been employed to check the results of the DNA study (Blumenthal, 2015). The difficulty in amplifying full-length barcodes from processed materials has led to the belief that it is effectively impossible to extract DNA from products made from extracts. However, it was shown by Kazi et al that it is possible to extract short fragments of DNA from a wide range of finished products including some made with extracts. This study was based on St John's Wort, *Hypericum perforatum.* The product range included capsules, tablets and also tinctures. In the study, 13 products were included and all 13 products were successful in yielding DNA that was verified to be *Hypericum perforatum* as labelled. It was however necessary to design a primer pair capable of amplifying an 80bp product in order to overcome the effects of DNA degradation during processing (Kazi et al., 2013).

The development of the PlantID assay, detailed in Chapter 3. Further development of the PlantID assay, yielded some good species-specific primers but not all of them were suitable to be used in a multiplex assay due to incompatible annealing temperatures. Due to the development and access to better technologies it has since become possible to run qPCR assays in tandem using different annealing temperatures. This has led to the development of qPCR assays for the detection of adulteration and or substitution of species of *Actaea* that could not be detected using PlantID. In the PlantID work it was not possible to obtain specific *Actaea dahurica* primers and this was because the vouchered DNA was found to be *Actaea simplex.* Vouchered DNA from another bank was sourced and the primers were trialled once again, this time proving to be specific for their intended target. In this work *A. racemosa*, *A. cimicifuga* and *A. dahurica* were concentrated upon as they were of the

highest interest. This is due to several occasions where Asian *Actaea* species have been found as adulterants or substitutions in Black Cohosh products (Jordan et al., 2010, Jiang et al., 2006).

Species-specific primer pairs were designed for use in singleplex qPCR assays for the three target species. The assays were first optimised using DNA from vouchered sources. Once this was completed, the assays were validated using reference samples which were sequenced to reinforce the results. Following on from the validation of the assay, commercially available finished products were tested. For legal reasons these products have been anonymised using a numerical naming system. In some cases, products were found to be adulterated or even substituted.

Importantly, an *Actaea* generic primer pair was utilised as a control in this assay. This was designed to amplify any *Actaea* species DNA present in a parallel reaction to the species-specific primers. For example, if there was no amplification of a template DNA with the *Actaea racemosa* primers but amplification occurred with the generic primer pair, it could be concluded that there was amplifiable DNA, but that it was not from *A. racemosa*. A negative result with the generic primers would indication the absence of any amplifiable DNA, and negative results from all of the species-specific primers would be expected.

Where possible the commercial samples were also sequenced to reinforce any results obtained but this was not straight forward when degraded DNA was present. The samples provided by the AHPA were also part of a chemical based analysis. The samples were analysed using mass spectroscopy and principle component analysis. These results added another layer of identification to the samples but were not always in agreement with the DNA based efforts.

The aims of this chapter are to:

- Utilise species-specific primers from the PlantID chapter for qPCR based assays (see Chapter 3. Further development of the PlantID assay).

- Demonstrate specificity of the primers using sequenced templates for *Actaea racemosa, Actaea dahurica* and *Actaea cimicifuga.*

- Validate the assay with reference samples (Identification reinforced in Chapter 2 Genetic analysis of Black Cohosh, *Actaea racemosa,* and potential adulterant species)

- Use the assay to test commercial products for the presence of the included species.

4.2 Materials and Method

4.2.1 Extraction of DNA using the Qiagen DNeasy Plant Mini Kit

For the full method please see: Section 3.2.2: Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit.

4.2.1.1 Extraction of DNA with additional protein digestion step

The Qiagen Dneasy Plant Mini Kit (Qiagen Inc., CA) and TissueLyser procedure was followed. To a 2 mL safe-lock centrifuge tube, 100 mg of wet plant material or 20 mg of dry material was added. A 3mm tungsten carbide bead was put inside the tube. The tubes were then placed into the TissueLyser adapter set, fixed to the clamps and ground for one minute at 30 Hz. The position of the tubes was then reversed and the previous step was repeated to ensure equal treatment. Then 630 µL of buffer AP1 supplemented with 600 µg of Proteinase K was added to each sample, and mixed thoroughly. To lyse the cells, the samples were then incubated for 12-18 hours at 42°C. The samples were mixed 2 to 3 times during the incubation by inversion.

The samples were incubated on ice for 5 minutes after the addition of 200 µL of buffer AP2.

The rest of the protocol is as in Section 3.2.2 Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit.

4.2.1.2 Extraction of DNA using the Qiagen Dneasy Plant Mini Kit with additional centrifugation step in initial material preparation stage

This step was made before extraction began. A few of the commercial samples were soft gel capsules and filled with a suspension of extract in oil. Two to three capsules were cut open using a sterile blade and the contents were placed inside a 2 mL centrifuge tube. The volume was typically around 100 µL. The tube was centrifuged for 10 minutes at full speed. The top oil layer was removed and the pellet of extract was used for the extraction process. The wet weight outlined in the protocol; 100 mg was used. The DNA was then extracted as outlined in section 3.2.2 Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit.

4.2.2 Standard (End Point) Polymerase Chain Reaction

For the full method please see section 2.2.5 Standard (End-point) Polymerase Chain Reaction.

4.2.3 Gel electrophoresis

For the full method please see section 3.2.9 Gel Electrophoresis using agarose.

4.2.4 Quantitative Polymerase Chain Reaction (qPCR)

For this method Sensifast™ qPCR Master Mix (Bioline, London) was used. This is a pre-prepared mix that just required the addition of primers, a template and nuclease free water. The Applied Biosystems StepOnePlus qPCR machine was used for cycling.

Table 30: The components and amounts required for a qPCR reaction

Component	Amount for 1 reaction (µL)
Sensifast™ 2x qPCR MasterMix	5
Forward primer	0.2
Reverse primer	0.2
Template	1
Nuclease free water	3.6

The following cycling conditions were employed:

Table 31: The cycling conditions required for amplification using Sensifast™ components for qPCR

1 cycle	40 cycles			Melt curve		
	Denature	**Anneal**	**Plate read**	**Start temperature**	**End temperature**	**Rate** (Plate read each incremental increase)
95°C	95°C	60°C/66°C		65°C	95°C	0.5°C/Min
5 minutes	15 seconds	1 minute				

4.3 Results and Discussion

4.3.1 Initial development of qPCR assays with vouchered DNA reference samples

Diluted high fidelity ITS PCR products were used as reference samples. Each DNA extraction used was also sequenced to ensure that the identification was correct. It was necessary to find a new *Actaea dahurica* template for these assays as it was shown in Chapter 2 that the DNA purchased from the Royal Botanic Gardens, Kew DNA Bank was in fact *Actaea simplex.* The specificity of the *A. dahurica* primers was checked again and is shown later in Figure 41. The specific primers produced a product with the new *A. dahurica* template. The new *A. dahurica* template was used to produce new sets of non-target panels for each of the other species. The specificity of the non *A. dahurica* species specific primers was checked again and no amplification was seen. This is also shown in Figure 41.

Table 32: Relevant information for target species DNA samples

Sample name	Sample number	Source	Labelled Species	Sequencing Results
Arac$^{-5.9}$	24092	Kew	*Actaea racemosa*	*Actaea racemosa*
Adah$^{-5.9}$	DB8499	BGBM	*Actaea dahurica*	*Actaea dahurica*
Acim-$^{5.9}$	10294	Kew	*Actaea cimicifuga*	*Actaea cimicifuga*

The aim for each assay was to see a positive result for the target template DNA and no amplification for the non-target panel. The non-target panel is outlined in Table 20 of Chapter 3. Further development of the PlantID assay. To recap, a non-target panel was made for each species included. This contained DNA of all species in the project except for the target. The non-target panels were

made up of 10 species templates and so a dilution of 10 times the target DNA was used to ensure the resulting concentration of each included species was that of the target DNA.

Foe each species, a qPCR experiment was performed to test specificity with this application. The results are shown in Figure 41.

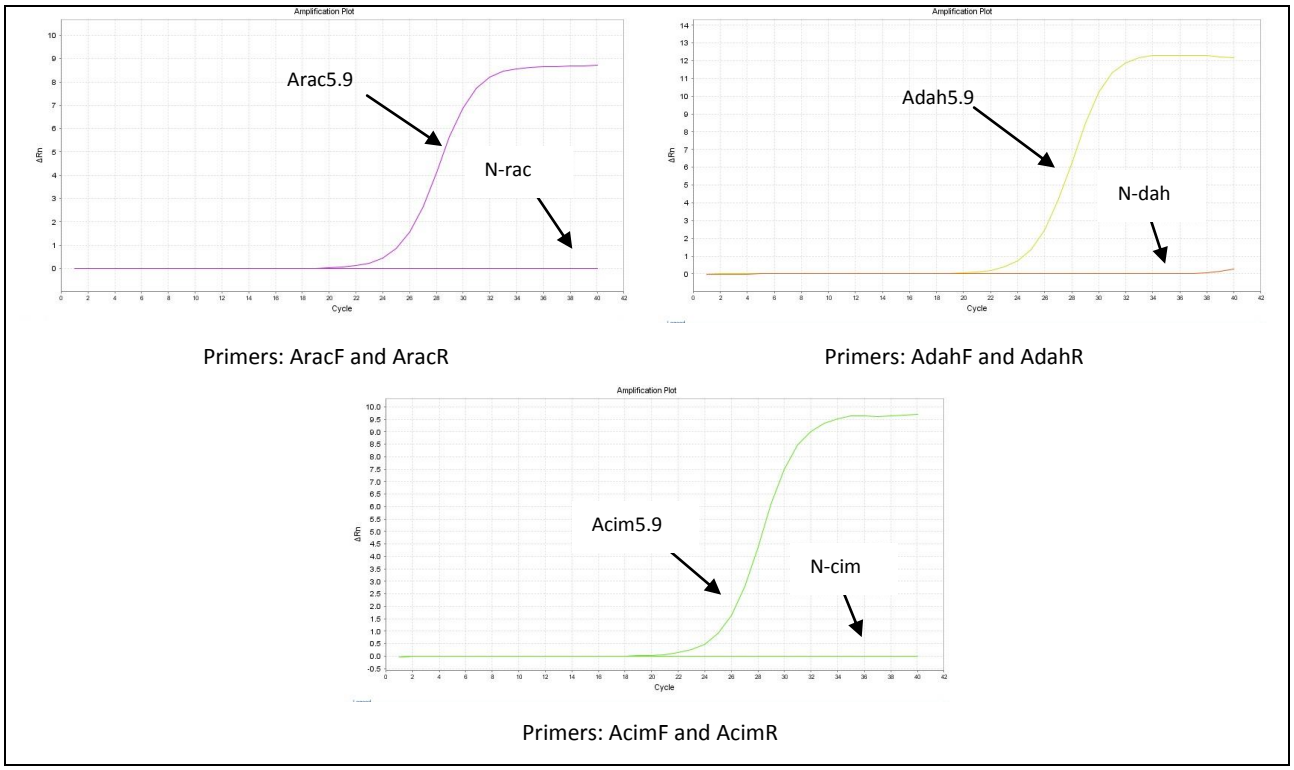


Figure 41: qPCR amplification plots showing positive amplification with the target templates and negative amplification with the non-target panel DNA

In Figure 41 each set of specific primers were used in qPCR reactions to amplify both the target template for the specific species and the non-target DNA panel for all species except the target. The aim was to see an amplification with the target DNA and amplification with a C_t of less than 35 (considered a negative result) for the non-target panels respectively for each species specific primer pair. For each set of species specific primers this was achieved.

To see how the assay would perform, some of the DNA extractions (6 initial samples) were tested with the *A. racemosa* specific primers. The results were at first difficult to interpret as is shown in Figure 42. It was difficult to see if a sample was negative for the presence of the species attempting to be detected or if the amount of DNA was low as there wasn't anything to compare to. It was only

possible to compare the samples within the experiment. Samples 1 to 3 looked positive but samples 4 to 6 had lower C_t values. This raised the question of how it was possible to tell which were positive and which were negative results. The difficulty was how to distinguish between positive reactions with a low concentration or poor quality templates and false positives resulting from low level primer mismatches when comparing against good quality templates.

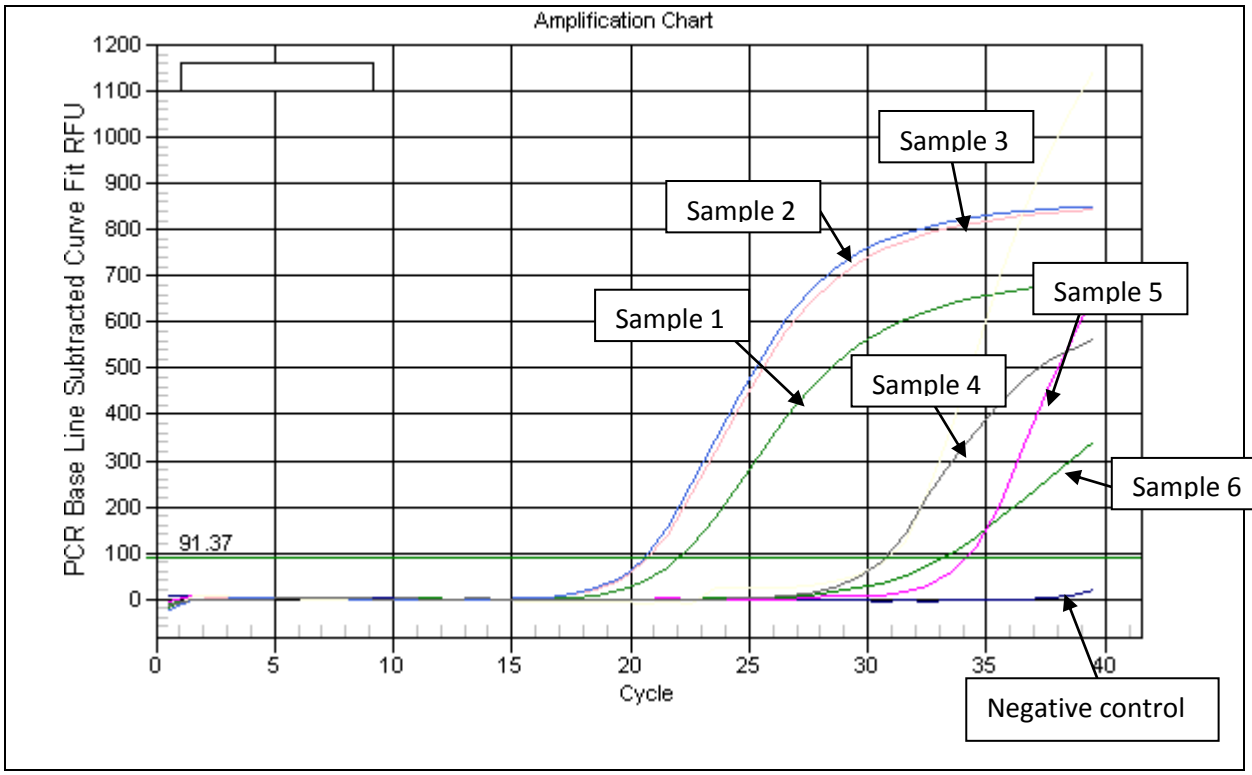


Figure 42: An amplification plot of *A. racemosa* specific species primer testing without generic primer controls

It showed that another primer pair was required as a control. This was termed a generic primer and was designed to amplify any and all of the *Actaea* species included in this work. A search of the primer sequence was performed using BLAST and it was found that the generic primer matches all *Actaea* species on the database. The results are shown in Table 33. The C_t value of the generic primer test also indicates the total number of amplifiable template molecules in the assay. Subtraction of this value from the C_t value of the species-specific test gives the ratio of species-specific template molecules to total template molecules when adjusted for the differences in efficiencies of the two primer pairs using a reference standard template. The results of amplification with this generic

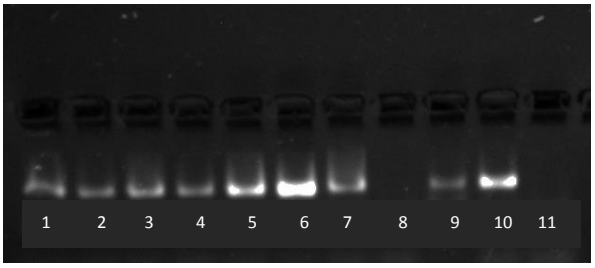
primer pair could be directly compared to the results of the species-specific primers test and therefore give an idea of how much DNA was present and how much of that DNA was a particular species. This overcame not being able to interpret the results of the species-specific tests alone. If the generic primer test was positive but all the species-specific primer tests were negative, it showed that another *Actaea* species was present and sequencing could be used to determine which one, if possible. The generic primer was used to amplify all the targets involved in the assays to ensure that it was working correctly. The primer pair was successful in being able to do this as shown in Figure 43.

The C_t value of the generic primer test also indicates the total number of amplifiable template molecules in the assay. Subtraction of this value from the C_t value of the species-specific test gives the ratio of species-specific template molecules to total template molecules when adjusted for the differences in efficiencies of the two primer pairs using a reference standard template. The results of amplification with this generic primer pair could be directly compared to the results of the species-specific primers test and therefore give an idea of how much DNA was present and how much of that DNA was a particular species. This overcame not being able to interpret the results of the species-specific tests alone.

Table 33: The matches obtained when using the generic primer sequence as a query in BLAST

Species match	Match to GenBank Sequence
Actaea racemosa	100%
Actaea cordifolia	100%
Actaea rubra	100%
Actaea pachypoda	100%
Actaea podocarpa	100%
Actaea heracleifolia	100%
Actaea dahurica	100%
Actaea simplex	100%
Actaea cimicifuga	100%
Actaea asiatica	100%
Actaea brachycarpa	100%
Actaea japonica	100%
Actaea frigida	100%
Actaea vaginata	100%

Actaea yunnanensis	100%
Actaea purpurea	100%
Actaea nanchuenensis	100%
Actaea mairei	100%
Actaea spicata	100%
Actaea elata	100%
Actaea biternata	100%
Actaea arizonica	100%
Actaea acerina	100%
Actaea laciniata	100%
Actaea kashmiriana	100%
Actaea europaea	100%
Actaea erythrocarpa	100%



1 – *Actaea racemosa*
2 – *Actaea pachypoda*
3 – *Actaea dahurica*
4 – *Actaea cimicifuga*
5 – *Actaea cordifolia*
6 – *Actaea rubra*
7 – *Actaea heracleifolia*
8 – *Caulophyllum thalictroides**
9 – *Actaea podocarpa*
10 – *Actaea simplex*
11 – No template control
** Intended not to amplify*

Figure 43: A gel to illustrate the ability of the generic primers to amplify any *Actaea* species but not samples from other genera.

The primer annealing temperatures were already optimised in the PlantID assay to maximise specificity so no further development was necessary in that respect. The primers were however tested over a range of concentrations to optimise amplification efficiency. When a new primer pair is planned for use in qPCR the efficiency should be analysed. This was carried out by making a serial dilution of the high fidelity ITS PCR products and then amplifying each dilution in triplicate. Three different concentrations of primers were trialled for each species set; 0.2 µM, 0.1 µM and 0.05 µM. Melt curve analysis was also included. The efficiencies of each set of primers is outlined in Figure 44 and the main aim was not just to choose a concentration that had an efficiency closest to 100% but also to choose efficiencies that were matched between the primer pairs, i.e. the generic primers and

species-specific primers to have the same efficiency so that they behave similar in reactions with the same template, and thus are comparable.

Table 34: The efficiencies obtained from analysing serial dilutions of target templates with species specific and generic primers.

	Primer efficiency (%)		
Primer pair target	0.2 µM	0.1 µM	0.05 µM
A. racemosa	89.654	97.716	84.797
A. cimicifuga	112.053	97.948	81.381
A. dahurica	100.319	96.164	80.290
Generic	115.339	97.525	54.357

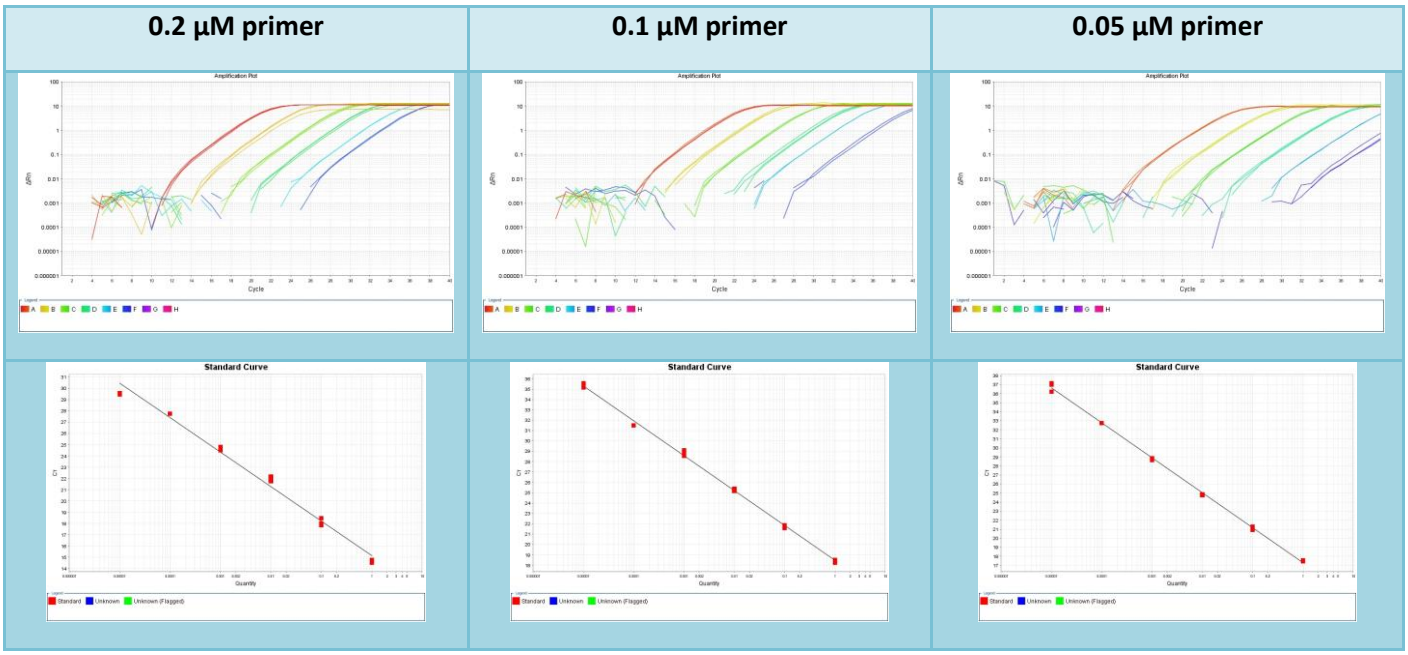
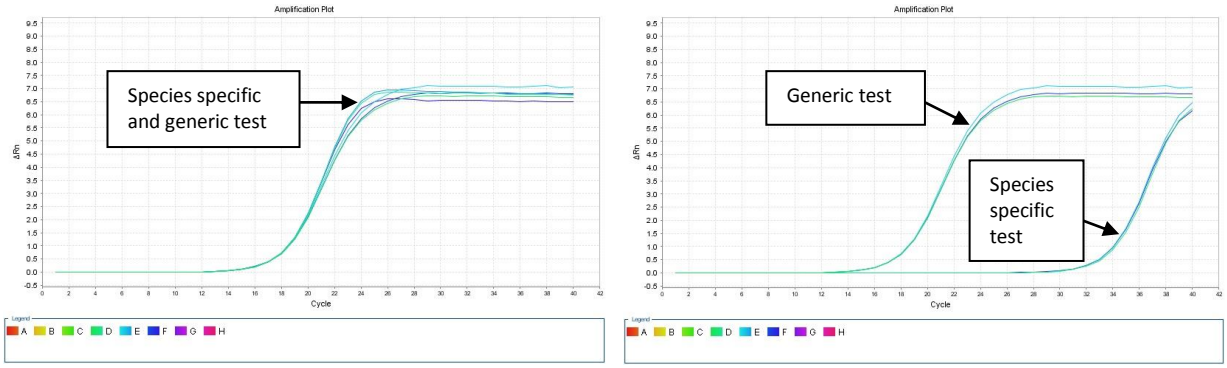


Figure 44: An example of optimisation for *A. racemosa* species specific primers – logarithmic amplification plots and standard curves shown

From the efficiency testing it was decided to use the concentration of 0.1 µM as this gave the most consistent efficiency across the primer pairs and was also as close to 100% as possible. The other concentrations either gave an over efficiency or were not efficient enough.

The next stage was to include the generic primers in a test with the reference samples. Tests were also set up with the non-target panel to illustrate how a negative test would look. This is shown in Figure 45. On the left hand side of the figure, the *A. racemosa* reference sample was used as a

template with the generic primer and *A. racemosa* specific primers. Both sets of primers show a positive result with a very similar C_t value for each primer pair. On the right hand side of the figure the *A. racemosa* non-target panel was used as a template. The generic primers have amplified the panel as *Actaea* species are present. The *A. racemosa* specific primers also show amplification of the non-target panel, but with a much higher C_t value than the generic test. The difference between the two C_t values (specific - generic) is around 16 cycles. Given the exponential nature of PCR amplification (the number of amplicons double each cycle at 100% efficiency of amplification), a 16-cycle difference corresponds to a difference between generic and specific template molecules of nearly 10^{-5}, which can be regarded as a negative result. Each sample was amplified in triplicate.



A positive result for a species specific test

A negative result for a species specific test

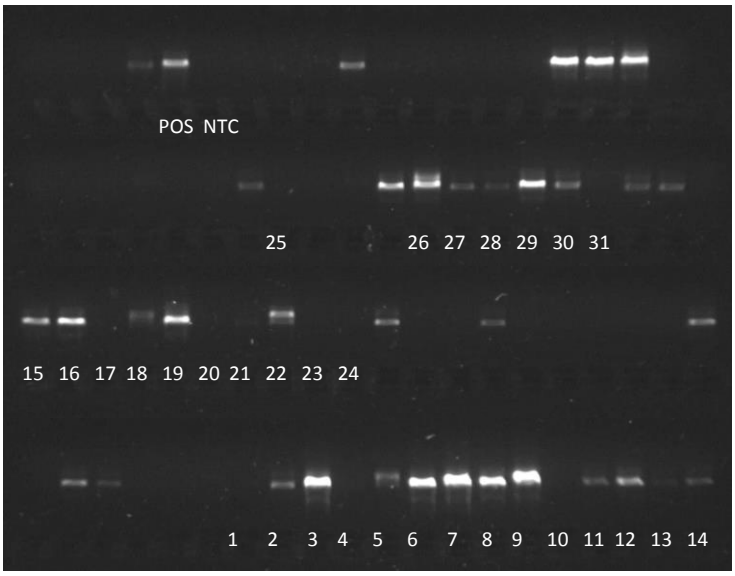
Figure 45: Examples of amplification plots for a positive and a negative test result

4.3.2 Validation of qPCR assays using reference samples coupled with sequencing

Samples were collected from many sources and sequencing was also performed to validate the identity. To start with, each of the DNA extracts from Table 35 was amplified with ITS1 and ITS4 primers using standard PCR. The resulting reactions were analysed using gel electrophoresis. A selection of the results is displayed in Figure 46. A bright crisp band indicated a good PCR product and these were selected for sequencing.

Table 35: A summary of information for validation samples used for the qPCR assays

Extraction number(s)	Implied species	Sample no.	Sample Material	Concentration (ng/µL)
S7	***Actaea racemosa***	AHPA-037	Cut Root and Rhizomes	50.4
1115	***Actaea racemosa***	AHPA-038	Cut Root and Rhizomes	18.9
1116	***Actaea racemosa***	AHPA-039	Cut Root and Rhizomes	24.8
1117	***Actaea racemosa***	AHPA-040	Cut Root and Rhizomes	37.4
1118	***Actaea racemosa***	AHPA-041	Cut Root and Rhizomes	25.3
S8	***Actaea racemosa***	AHPA-042	Cut Root and Rhizomes	14.2
S9	***Actaea racemosa***	AHPA-043	Powdered Root and Rhizome	64.6
1119	***Actaea cimicifuga***	AHPA-044	Whole Raw Material	39.9
1111	***Actaea racemosa***	AHPA-045	Whole Raw Material	33.0
S10	***Actaea racemosa***	AHPA-046	Whole Raw Material	12.8
1120	***Actaea racemosa***	AHPA-047	Whole Raw Material	40.2
S21	***Actaea racemosa***	AHPA-052	Powdered Raw Material	6.2
S22	***Actaea racemosa***	AHPA-053	Powdered Raw Material	9.0
S23	***Actaea racemosa***	AHPA-056	Powdered Raw Material	16.1
S24	***Actaea racemosa***	AHPA-057	Powdered Raw Material	11.2
S25	***Actaea dahurica***	AHPA-058	Whole Raw Material	33.2
S26	***Actaea racemosa***	AHPA-059	Extract	3.0
S27	***Actaea racemosa***	AHPA-061	Whole Raw Material	9.3
S11	***Actaea racemosa***	AHPA-062	Whole Raw Material	27.4
S28	***Actaea dahurica***	AHPA-063	Whole Raw Material	10.4
S12	***Actaea racemosa***	AHPA-064	Whole Reference sample	37.8
S13	***Actaea racemosa***	AHPA-065	Whole Reference sample	76.0
S17	***Actaea racemosa***	AHPA-066	Whole Reference sample	4.7
S18	***Actaea racemosa***	AHPA-067	Whole Reference sample	4.9
S14	***Actaea racemosa***	AHPA-068	Whole Reference sample	31.9
S19	***Actaea racemosa***	AHPA-069	Whole Reference sample	7.1
S20	***Actaea racemosa***	AHPA-071	Whole Reference sample	17.3
S15	***Actaea pachypoda***	AHPA-078	Whole Raw Material	124.3
BC050	***Actaea racemosa***	DR10-014-A	Whole Raw Material	62.3
BC046	***Actaea cimicifuga***	Ch.B.13884K116-A	Whole Raw Material	35.8
BC049	***Actaea dahurica***	Ch.B.01230952	Whole Raw Material	31.9



1-S7, 2-S8, 3-S9, 4-S10, 5-S11, 6-S12, 7-S13, 8-S14, 9-S15, 10-S16, 11-S17, 12-S18, 13-S19, 14-S20, 15-S21, 16-S22, 17-S23, 18-S24, 19-S25, 20-S26, 21-S27, 22-S28, 23-S29, 24-S30, 25-1111, 26-1115, 27-1116, 28-1117, 29-1118, 30-1119, 31-1120.

Pos – Positive control, NTC – No template control

Figure 46: An agarose gel to show the presence-absence of a PCR product using ITS primers for a selection of samples

Each sample was amplified with the generic primers, *A. racemosa* specific primers, *A. cimicifuga* specific primers and *A. dahurica* specific primers. The qPCR machine could analyse 96 tubes in each run and so for each run a set of controls were included for each set of primers used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Arac	Arac	Arac	7	7	7	Arac	Arac	Arac	7	7	7
B	N-Arac	N-Arac	N-Arac	8	8	8	N-Arac	N-Arac	N-Arac	8	8	8
C	1	1	1	9	9	9	1	1	1	9	9	9
D	2	2	2	10	10	10	2	2	2	10	10	10
E	3	3	3	11	11	11	3	3	3	11	11	11
F	4	4	4	12	12	12	4	4	4	12	12	12
G	5	5	5	13	13	13	5	5	5	13	13	13
H	6	6	6	B	B	B	6	6	6	B	B	B

Figure 47: A typical layout of the 96 tube plate used for qPCR testing – *A. racemosa* species-specific test (Pink) and generic test shown as an example (Blue).

In Figure 47 a typical qPCR test plate is displayed. The positive control is required as a comparison for the test samples. In a qPCR test, results are given as C_t (threshold cycle) values. This is the cycle number when the detected fluorescence exceeds the threshold set out for the reaction. The C_t value of the specific test is subtracted from the C_t value of the generic test for the positive control. The same is calculated for each of the test samples. The resulting figure of the positive sample is subtracted from the test sample. If this number is 0 (+/-1.0) it is a clear detection for the species that is being tested for. This was chosen as it indicates that the variation is less than 2 fold difference between the two primers. More than 1.1-6.0 is not as clear and a further test such as sequencing may be required to back up the result. It is likely that values that exceed 1 by a marginal amount are still indicative of a positive result, but the test replicates may have some variation. Any figure more than 6.1 is clearly negative for that species detection. This was chosen as exceeding this number suggests a 100 fold difference in effective template concentration between the two assays. In quality control terms this is regarded as a negative result as it falls below the threshold of contamination. These values are described as $\Delta\Delta C_t$ and the equation is shown below:

$\mathbf{\Delta\Delta C_t}$: $[(C_t \text{ specific} - C_t \text{ generic})_{sample} - (C_t \text{ specific} - C_t \text{ generic})_{reference}]$.

Table 36: The C_t values of validation samples and controls (Denoted with * with each set of controls displayed beneath the samples from the same experiment)

Sample number	Extract Number	*A. racemosa* test AVG C_t		*A. dahurica* test AVG C_t		*A. cimicifuga* test AVG C_t	
		Generic	*A. racemosa*	Generic	*A. dahurica*	Generic	*A. cimicifuga*
AHPA-BC042	S8	19.84	18.71	17.25	32.25	19.84	26.64
AHPA-BC064	S12	18.13	16.12	15.71	32.03	18.13	28.77
AHPA-BC065	S13	15.92	12.85	13.35	30.98	15.92	22.79
AHPA-BC068	S14	18.64	16.54	16.03	29.90	18.64	27.32
AHPA-BC078	S15	16.29	19.10	13.85	28.62	16.29	23.38
AHPA-BC052	S21	21.23	19.13	18.53	35.55	21.23	26.15
AHPA-BC053	S22	21.45	19.64	19.23	34.69	21.44	31.08
AHPA-BC058	S25	16.98	22.09	14.36	13.50	16.98	22.79
*A. racemosa**	Arac5.9	25.60	24.79	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	23.31	25.37	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	23.60	19.38
AHPA-BC039	1116	17.39	16.52	17.26	34.30	17.39	28.17
AHPA-BC044	1119	15.72	25.94	18.02	22.90	15.72	13.89
*A. racemosa**	Arac5.9	24.32	24.93	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	24.47	23.66	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	24.66	23.19
AHPA- BC040	1117	19.78	19.60	15.01	35.83	19.78	27.92
AHPA- BC041	1118	25.28	24.75	20.22	ND	25.28	29.77
AHPA-BC043	S9	17.05	15.97	12.61	33.23	17.05	25.04
*A. racemosa**	Arac5.9	28.69	28.78	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	23.31	25.37	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	25.61	23.81
AHPA- BC063	S28	19.42	26.57	16.75	16.51	19.42	26.58
AHPA-BC066	S17	20.14	18.01	18.26	34.70	20.14	28.86
AHPA-BC067	S18	18.95	17.05	16.55	38.46	18.98	27.95
AHPA-BC069	S19	22.49	20.62	19.81	35.86	22.49	30.13
AHPA- BC071	S20	20.75	17.92	18.47	ND	20.75	29.56
AHPA-BC057	S24	32.21	28.75	29.39	ND	32.21	30.23
AHPA-BC038	1115	17.29	14.71	14.93	34.35	17.29	24.61
*A. racemosa**	Arac5.9	25.60	24.79	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	23.31	25.37	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	23.60	19.38
DR10-014-A	BC050	**13.39**	**11.74**	**9.14**	**27.18**	**13.39**	**23.13**
Ch.B.01230952	BC049	**18.91**	**28.01**	**13.86**	**13.84**	**18.91**	**20.69**
Ch.B.13884K116-A	BC046	**17.38**	**32.23**	**12.61**	**12.25**	**17.38**	**14.99**
*A. racemosa**	Arac5.9	**28.69**	**28.78**	**N/A**	**N/A**	**N/A**	**N/A**
*A. dahurica**	Adah5.9	**N/A**	**N/A**	**23.31**	**25.37**	**N/A**	**N/A**
*A. cimicifuga**	Acim5.9	**N/A**	**N/A**	**N/A**	**N/A**	**25.61**	**23.81**

Table 36 shows the positive controls used in each assay with an (*). These are located below each set of results from an experiment. C_t values of more than 35 are deemed as a negative result.

Table 37: The results of specific qPCR assay testing with validation samples – illustrating the capability of the assay.

Commercial Sample	DMU Extract number	Barcode sequence	*A. racemosa* qPCR $\Delta\Delta C_t$	*A. dahurica* qPCR $\Delta\Delta C_t$	*A. cimicifuga* qPCR $\Delta\Delta C_t$	Conclusions
AHPA-BC038	**1115**	*A. racemosa*	-1.8	17.4	11.5	Likely *A. racemosa*
AHPA-BC039	**1116**	*A. racemosa*	-1.5	17.9	12.3	*A. racemosa*
AHPA-BC040	**1117**	*A. racemosa*	-0.3	18.8	10.0	*A. racemosa*
AHPA-BC041	**1118**	-	-0.6	17.7	6.3	Likely *A. racemosa*
AHPA-BC042	**S8**	*A. racemosa*	-0.3	15.8	8.6	*A. racemosa*
AHPA-BC043	**S9**	*A. racemosa*	-1.2	18.6	9.8	*A. racemosa*
AHPA-BC044	**1119**	*A. cimicifuga*	9.6	8.3	-0.4	*A. cimicifuga*
AHPA-BC052	**S21**	*A. racemosa*	-1.3	17.8	6.7	*A. racemosa*
AHPA-BC053	**S22**	*A. racemosa*	-1.0	16.3	11.4	*A. racemosa*
AHPA-BC057	**S24**	-	-2.7	8.6	2.2	Possibly *A. racemosa* and *A. cimicifuga*
AHPA-BC058	**S25**	*A. dahurica*	6.0	-0.05	7.6	*A. dahurica*
AHPA-BC063	**S28**	*A. dahurica*	8.0	-2.3	11.4	Likely *A. dahurica*
AHPA-BC064	**S12**	*A. racemosa*	-1.2	17.1	12.4	*A. racemosa*
AHPA-BC065	**S13**	*A. racemosa*	-2.2	18.4	8.6	*A. racemosa*
AHPA-BC066	**S17**	*A. racemosa*	-1.3	14.4	13.0	*A. racemosa*
AHPA-BC067	**S18**	*A. racemosa*	-1.1	19.9	13.2	*A. racemosa*
AHPA-BC068	**S14**	*A. racemosa*	-1.3	14.7	10.4	*A. racemosa*
AHPA-BC069	**S19**	*A. racemosa*	-1.1	14.0	11.9	*A. racemosa*
AHPA-BC078	**S15**	*A. pachypoda*	3.7	15.6	8.9	*A. pachypoda*
DR10-014-A	**BC050**	*A. racemosa*	-1.7	-16.0	-11.6	*A. racemosa*
Ch.B.01230952	**BC049**	-	-9.0	2.1	3.6	*A. dahurica* and possibly *A. cimicifuga* present
Ch.B.13884K116-A	**BC046**	*A. cimicifuga*	-14.8	-2.4	-0.6	*A. cimicifuga* and possibly *A. dahurica* present

$\Delta\Delta C_t$: $[(C_t \text{ specific} - C_t \text{ generic})_{sample} - (C_t \text{ specific} - C_t \text{ generic})_{reference}]$. A value of 0.0 +/-1.0 is a clear positive result. A value > 6.0 is a clear negative.

The barcode sequencing results are outlined in Table 38 and give further details about why and how the identification has been chosen.

Table 38: A summary of the BLAST results for validation samples – top hits shown

Sample number	Extraction number	Region sequenced	Sequence match	Accession number of top match	Match Percentage	MOTU
AHPA-BC038	**1115**	*matK*	*A. racemosa*	KU662878.1	100%	N/A
AHPA-BC039	**1116**	ITS	*A. racemosa*	AB987687.1	99%	*A. racemosa*
AHPA-BC040	**1117**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC042	**S8**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC043	**S9**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC044	**1119**	ITS	*A. cimicifuga*	GQ351362.1	99%	*A. cimicifuga*
AHPA-BC052	**S21**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC053	**S22**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC058	**S25**	ITS	*A. dahurica*	AB987685.1	98%	*A. dahurica*
AHPA-BC063	**S28**	*matK*	*A. dahurica*	KU662876.1	98%	N/A
AHPA-BC064	**S12**	ITS	*A. racemosa*	GQ409511.1	98%	*A. racemosa*
AHPA-BC065	**S13**	ITS	*A. racemosa*	GQ409509.1	98%	*A. racemosa*
AHPA-BC066	**S17**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC067	**S18**	ITS	*A. racemosa*	AB987687.1	95%	*A. racemosa*
AHPA-BC068	**S14**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
AHPA-BC069	**S19**	*matK*	*A. racemosa*	KU662878.1	99%	N/A
AHPA-BC078	**S15**	ITS	*A. pachypoda*	Z98277.1	99%	*A. pachypoda*
DR10-014-A	**BC050**	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
Ch.B.13884K116-A	**BC046**	ITS	*A. dahurica* *A. cimicifuga*	GQ351361.1 JQ033518.1	81% 87%	*A. dahurica* *A. cimicifuga*

Table 38 shows the sequencing results and how they matched to available sequences from the GenBank database. It also gives the MOTU species, which is the branch in the DNA tree diagram shown in Chapter 2; Figure 17, Figure 18 and Figure 19. It was not possible to group the *MatK* sequences into a tree with distinguishable branches and in these cases the MOTU box has been filled with N/A. These sequences were used in cases where a successful ITS sequence was not achievable. They are not as reliable and were only used to backup other results.

4.3.3 Testing of commercial Black Cohosh products with the developed qPCR assays

The first step in this section was to extract DNA from the commercial products. From past experience of finding it challenging to extract good quality DNA from these kinds of products, it was decided to use a modified method of extraction. Baker et al (2012) discovered that a prolonged protein digestion step was useful and so this was used (Baker et al., 2012). Many of the samples also required an isopropanol clean up. Table 39 gives a summary of sample type, extraction numbers and quantification data.

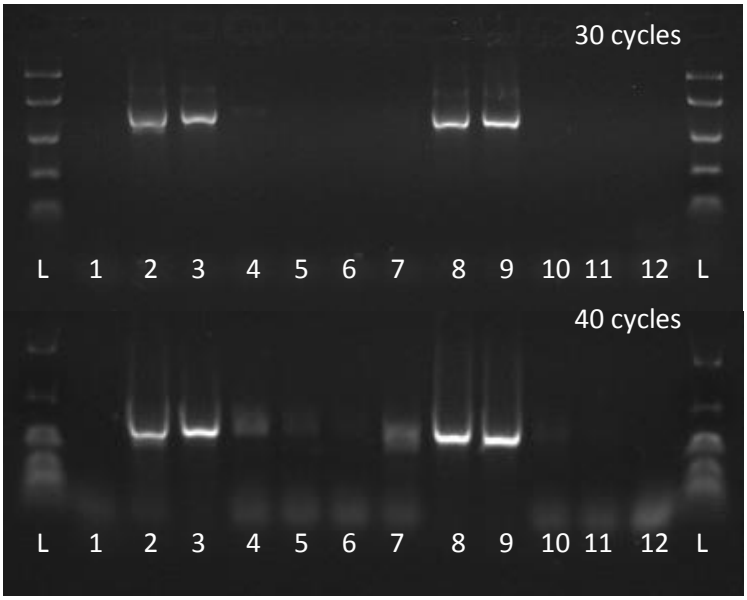
Table 39: Sample information of commercial product DNA extracts

DNA extraction no.	Labelled species	Sample no.	Sample Material	Composition	DNA Concentration (µg/mL)
BC023	*Actaea racemosa*	103	Capsule, ground raw root	2.5% total triterpene glycosides – 200 mg	2.07
BC024	*Actaea racemosa*	104	Capsule, extract and ground raw root	2.5% total triterpene glycosides – 200 mg, Root powder 100 mg	6.48
BC025	*Actaea racemosa*	105	Capsule, extract and ground raw root	2.5% total triterpene glycosides – 200 mg, Root powder 100 mg	5.22
BC026	*Actaea racemosa*	106	Capsule, ground raw root	200 mg root powder, 200 mg extract standardised 2.5% total triterpene glycosides 27-deoxyactein 5 mg	8.08
BC027	*Actaea racemosa*	107	Soft gel capsule, extract	Black Cohosh Extract 160 mg, Root and Rhizome, 2.5% total triterpene glycosides, 27-deoxyactein 4 mg	0.08
BC032	*Actaea racemosa*	107	Soft gel capsule, extract	Black Cohosh Extract 160 mg, Root and Rhizome, 2.5% total triterpene glycosides, 27-deoxyactein 4 mg	1.36
BC028	*Actaea racemosa*	108	Soft gel capsule, extract	Black Cohosh Extract 160 mg, Root and Rhizome, 2.5% total triterpene glycosides, 27-deoxyactein 4 mg	1.59
BC033	*Actaea racemosa*	108	Soft gel capsule, extract	Black Cohosh Extract 160 mg, Root and Rhizome, 2.5% total triterpene glycosides, 27-deoxyactein 4 mg	2.91
BC029	*Actaea racemosa*	109	Coated caplet, extract	2.5% total triterpene glycosides – 50 mg. (Also containing Soy, Vitex, Dong Quai, Liquorish)	1.64
BC030	*Actaea racemosa*	120	Capsule, extract and ground raw root	2.5% total triterpene glycosides – 200 mg, Root powder 100 mg	5.62
BC031	*Actaea racemosa*	121	Capsule, extract and ground raw root	2.5% total triterpene glycosides – 200 mg, Root powder 100 mg	6.02
BC038	*Actaea racemosa*	080	Capsule, extract	Black Cohosh extract, 30 mg	9.50
BC039	*Actaea racemosa*	081	Capsule, powdered root	Powdered Root, 200 mg	16.70
BC040	*Actaea racemosa*	082	Capsule, extract	Black Cohosh (root) 2.5% total triterpene glycosides – 100 mg	2.60
BC041	*Actaea racemosa*	083	Tablet, extract	Black Cohosh Root Extract 200 mg equivalent to dried root 500 mg, 5mg triterpene glycosides	2.70
BC042	*Actaea racemosa*	084	Capsule, root	Black Cohosh Root 540 mg	6.90
BC043	*Actaea racemosa*	085	Soft Gel Capsule, extract	Black Cohosh Extract 160 mg, Root and Rhizome, 2.5% total triterpene glycosides, 27-deoxyactein 4 mg	16.90

BC044	*Actaea racemosa*	086	Film coated tablet, extract – THR registered	29.25-55.25mg Black Cohosh, 6.5 mg extract	3.90
BC045	*Actaea racemosa*	087	Film coated tablet, extract – THR registered	29.25-55.25mg Black Cohosh, 6.5 mg extract	35.10
1032	*Actaea racemosa*	AHPA-BC001	Tea bag	Powdered Root – 1250 mg	49.00
1093	*Actaea racemosa*	AHPA-BC002	Capsules, root	Powdered Root – 540 mg	24.30
1105	*Actaea racemosa*	AHPA-BC003	Capsules, root	Powdered Root – 540 mg	21.70
1106	*Actaea racemosa*	AHPA-BC004	Capsules, root	Powdered Root – 540 mg	15.50
1094	*Actaea racemosa*	AHPA-BC005	Capsules, root	Powdered Root – 540 mg	21.20
S1	*Actaea racemosa*	AHPA-BC006	Capsules, root	Powdered Root – 410 mg	23.00
1095	*Actaea racemosa*	AHPA-BC007	Capsules, root	Powdered Root –300 mg	19.90
1096	*Actaea racemosa*	AHPA-BC008	Capsules, root	Powdered Root – 540 mg	16.00
1097	*Actaea racemosa*	AHPA-BC009	Capsules, root	Powdered Root – 540 mg	14.50
1098	*Actaea racemosa*	AHPA-BC010	Capsules, root	Powdered Root – 540 mg	2.20
1099	*Actaea racemosa*	AHPA-BC011	Capsules, root	Powdered Root – 100 mg	4.90
1100	*Actaea racemosa*	AHPA-BC012	Capsules, root	Powdered Root – 540 mg	6.50
1101	*Actaea racemosa*	AHPA-BC013	Capsules, root	Powdered Root – 370 mg	28.50
S2	*Actaea racemosa*	AHPA-BC014	Capsules, root	Powdered Root – 540 mg	23.60
1107	*Actaea racemosa*	AHPA-BC015	Capsules, root	Powdered Root – 200 mg	9.30
1108	*Actaea racemosa*	AHPA-BC016	Capsules, root	Powdered Root – 540 mg	2.40
1109	*Actaea racemosa*	AHPA-BC017	Capsules, root and extract	530 mg root, 20 mg extract standardized to min. 5% triterpene glycosides calculated as 26-deoxyactein	12.10
1110	*Actaea racemosa*	AHPA-BC018	Capsules, root and extract	545 mg (No Break Down Provided)	16.80
S3	*Actaea racemosa*	AHPA-BC019	Capsules, root and extract	185 mg root, 40 mg extract standardized to 2.5% triterpene glycosides	30.50
S4	*Actaea racemosa*	AHPA-BC021	Capsules, root and extract	380 mg root, 8 mg extract standardized to 2.5% triterpene glycosides	21.10
1112	*Actaea racemosa*	AHPA-BC022	Capsules, root and extract	545 mg (No Break Down Provided)	43.90
1113	*Actaea racemosa*	AHPA-BC023	Capsules, extract	100 mg, extract standardized to 2.5% triterpene glycosides	10.90
1114	*Actaea racemosa*	AHPA-BC026	Capsules, extract	40 mg, extract standardized to 2.5% triterpene glycosides calculated as 26-deoxyactein	4.40
S5	*Actaea racemosa*	AHPA-BC028	Capsules, extract	135 mg (4:1 extract) + 0.04 (extract standardized to 2.5% triterpene glycosides)	1.60
S6	*Actaea racemosa*	AHPA-BC031	Capsules, extract	40 mg, extract standardized to 2.5% triterpene glycosides calculated as 26-deoxyactein	1.20
S7	*Actaea racemosa*	AHPA-BC032	Capsules, extract	250 mg, extract standardized to 2.5% triterpene glycosides	50.40

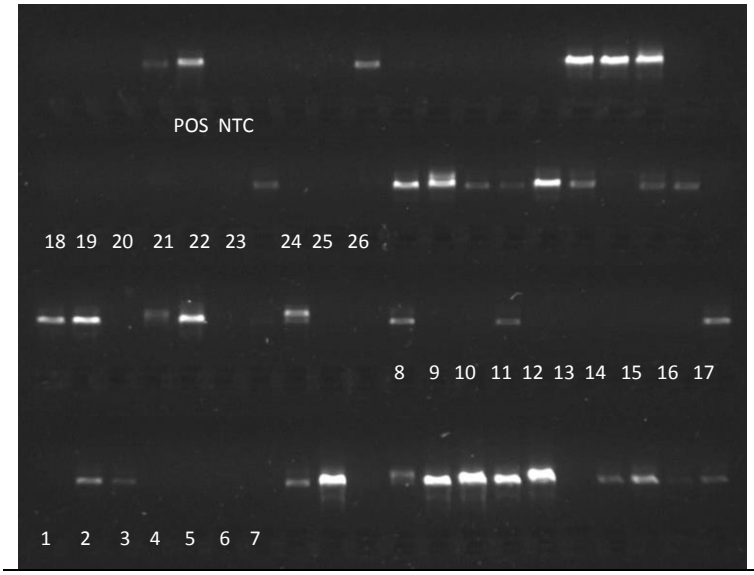
As can be seen from the DNA quantification results the amounts of DNA that were yielded were often low. It was however possible to work with these as can be seen with the ITS PCR results shown in Figure 48. It was found at 30 cycles that not many products yielded a PCR product. With 40 cycles

this showed that some of the other products had a small amount of useable DNA present, which will be detected in the qPCR assay as 40 cycles were also used.



L – DNA ladder
1 – BC023
2 – BC024
3 – BC025
4 – BC026
5 – BC027
6 – BC028
7 – BC029
8 – BC030
9 – BC031
10 – BC032
11 – BC033
12 – No template control

Figure 48: ITS PCRs of a selection of the commercial product samples at 30 cycles and 40 cycles respectively



1-S1, 2-S2, 3-S3, 4-S4, 5-S5, 6-S6, 7-S7, 8-1032, 9-1093, 10-1094, 11-1095, 12-1096, 13-1097, 14-1098, 15-1099, 16-1100 17-1101, 18-1105, 19-1106, 20-1107, 21-1108, 22-1109, 23-1110, 24-1112, 25-1113, 26-1114.

Pos – Positive control, NTC – No template control

Figure 49: An agarose gel to show the presence-absence of PCR products with ITS primers – commercial products

The C_t values of all the individual tests are outlined in Table 40 for information purposes and the $\Delta\Delta C_T$ values are outlined in Table 41.

Table 40: C_t values of species-specific and generic primer tests with commercial samples (Controls shown with *)

Sample number	Extract Number	*A. racemosa* test AVG C_t		*A. dahurica* test AVG C_t		*A. cimicifuga* test AVG C_t	
		Generic	*A. racemosa*	Generic	*A. dahurica*	Generic	*A. cimicifuga*
AHPA-BC001	1032	15.58	24.36	19.11	15.14	16.62	22.40
AHPA-BC002	1093	26.38	27.39	31.88	28.04	28.43	34.48
AHPA-BC003	1105	22.56	22.22	26.81	29.42	24.11	ND
AHPA-BC004	1106	26.76	26.14	32.32	30.26	28.97	ND
AHPA-BC005	1094	26.70	27.42	31.76	34.97	28.76	ND
AHPA-BC006	S1	26.36	26.72	35.77	ND	32.50	39.86
AHPA-BC007	1095	15.94	16.51	20.34	34.31	17.90	33.33
AHPA-BC008	1096	17.86	19.31	22.29	32.18	19.46	31.91
AHPA-BC009	1097	26.34	27.59	30.62	32.20	28.18	36.26
*A. racemosa**	Arac5.9	13.69	14.40	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	24.77	21.27	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	20.40	20.81
AHPA-BC010	1098	31.84	ND	32.78	31.05	31.84	36.17
AHPA-BC011	1099	35.49	34.93	34.09	29.71	35.49	35.22
AHPA-BC012	1100	34.48	36.99	33.84	31.39	34.48	ND
AHPA-BC013	1101	17.18	21.14	19.53	30.15	17.18	23.33
AHPA-BC014	S2	16.51	21.14	30.23	ND	16.51	23.09
AHPA-BC015	1107	29.34	35.93	25.54	ND	25.54	36.94
AHPA-BC016	1108	32.96	ND	28.55	ND	28.55	31.91
*A. racemosa**	Arac5.9	17.94	21.44	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	20.23	26.96	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	18.67	23.75
AHPA-BC019	S3	22.50	22.72	20.04	27.91	22.50	30.33
*A. racemosa**	Arac5.9	28.69	28.78	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	23.31	25.37	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	25.61	23.81
103	BC023	28.8	31.0	31.5	29.9	28.8	27.2
104	BC024	20.30	19.4	22.8	ND	20.3	31.6
105	BC025	18.6	17.8	20.9	29.4	18.6	30.1
106	BC026	28.4	28.6	30.7	30.3	28.4	33.2
107	BC027	29.5	29.5	32.4	32.6	29.5	30.9
108	BC028	30.3	31.6	33.6	35.5	30.3	32.7
*A. racemosa**	Arac5.9	24.5	23.4	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	26.7	23.2	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	22.2	20.2
109	BC029	31.1	33.0	33.1	ND	31.1	37.1
120	BC030	21.1	18.1	22.9	32.5	21.1	33.8
121	BC031	18.2	15.4	20.1	36.5	18.2	30.4
*A. racemosa**	Arac5.9	24.3	24.9	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	26.7	23.2	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	24.7	23.2
080	BC038	28.68	32.25	27.49	34.79	28.68	39.10
081	BC039	12.49	15.46	11.62	28.78	12.49	23.01
082	BC040	26.56	29.60	25.19	31.36	26.56	30.02
083	BC041	34.75	34.76	31.39	ND	34.75	ND
084	BC042	27.54	33.63	26.40	35.07	27.54	35.38
085	BC043	22.38	25.95	21.01	30.97	22.38	28.82
086	BC044	33.56	ND	33.83	ND	33.56	ND
087	BC045	34.84	36.08	34.25	36.72	34.84	33.79
*A. racemosa**	Arac5.9	23.86	26.09	N/A	N/A	N/A	N/A
*A. dahurica**	Adah5.9	N/A	N/A	23.80	28.22	N/A	N/A
*A. cimicifuga**	Acim5.9	N/A	N/A	N/A	N/A	20.66	17.86

Table 41: The results of the species specific tests showing $\Delta\Delta C_t$ values – commercial products

Commercial Sample	Chemistry results	DMU Extract number	Barcode sequence	*A. racemosa* qPCR $\Delta\Delta C_t$	*A. dahurica* qPCR $\Delta\Delta C_t$	*A. cimicifuga* qPCR $\Delta\Delta C_t$	Conclusions
AHPA-BC001	*A. cimicifuga*	1032	*Actaea dahurica*	8.1	-0.5	5.4	*A. dahurica*
AHPA-BC002	*A. racemosa*	1093	-	0.3	-0.3	5.6	*A. racemosa* and *A. dahurica*
AHPA-BC003	*A. racemosa*	1105	*Actaea racemosa*	-1.0	6.1	15.5	*A. racemosa*
AHPA-BC004	*A. racemosa*	1106	-	-1.3	1.4	10.6	*A. racemosa* and possibly *A. dahurica*
AHPA-BC005	*A. racemosa*	1094	-	0.01	6.7	10.8	*A. racemosa*
AHPA-BC006	*Astilbe biternata/ A. dahurica*	S1	-	-0.4	7.7	7.0	*A. racemosa*
AHPA-BC007	*A. racemosa*	1095	*Actaea racemosa*	-0.1	17.5	15.0	*A. racemosa*
AHPA-BC008	*A. racemosa*	1096	*Actaea racemosa*	0.7	13.4	12.0	*A. racemosa*
AHPA-BC009	*A. racemosa*	1097	-	0.5	5.1	7.7	*A. racemosa*
AHPA-BC010	*Serratula chinensis*	1098	-	4.7	1.8	3.9	Possibly *A. dahurica*
AHPA-BC011	*A. racemosa*	1099	-	-4.1	-0.9	-0.7	NOT *A. racemosa*. Possible *A. dahurica* or *A. cimicifuga*
AHPA-BC012	*A. cimicifuga*	1100	-	-1.0	1.0	5.1	NOT *A. cimicifuga*. Possible *A. dahurica* or *A. racemosa*
AHPA-BC013	*A. racemosa*	1101	*Actaea racemosa*	0.5	14.1	5.7	*A. racemosa*
AHPA-BC014	*A. racemosa*	S2	*Actaea racemosa*	1.1	13.3	6.2	*A. racemosa*
AHPA-BC015	*A. racemosa*	1107	**-**	4.0	7.7	6.3	NOT *A. dahurica* or *A. cimicifuga*
AHPA-BC016	*A. dahurica*	1108	**-**	3.5	4.7	-1.7	NOT *A. dahurica* or *A. racemosa.* Possible *A. cimicifuga*
AHPA-BC019	-	**S3**	*Actaea racemosa*	0.1	5.8	9.6	*A. racemosa*
103	-	**BC023**	**-**	1.6	1.9	3.6	Likely *A. racemosa* and *A. dahurica*
104	-	**BC024**	**-**	0.2	ND	13.3	*A. racemosa*
105	-	**BC025**	***Actaea racemosa***	0.9	12	13.5	*A. racemosa*
106	-	**BC026**	**-**	1.3	3.1	6.8	*A. racemosa*
107	-	**BC027**	***Actaea racemosa***	1.1	3.7	3.4	Contains *A. racemosa.*
108	-	**BC028**	**-**	2.4	1.6	4.4	Possibly contains *A. dahurica* and *A. racemosa*
109	-	**BC029**	**Not able to sequence as polyherbal**	1.3	ND	7.5	Contains *A. racemosa*

120	-	**BC030**	-	-3.6	13.1	14.2	*A. racemosa*
121	-	**BC031**	-	-3.4	19.9	13.7	*A. racemosa*
080	-	**BC038**	-	1.3	2.9	13.2	Possibly contains *A. racemosa* and *A. dahurica*
081	-	**BC039**	***Actaea racemosa***	0.7	12.8	13.3	*A. racemosa*
082	-	**BC040**	***Actaea racemosa***	0.8	1.8	6.3	Contains *A. racemosa* and Possibly *A. dahurica*
083	-	**BC041**	-	-2.2	ND	ND	Possibly *A. racemosa*
084	-	**BC042**	-	3.9	4.3	10.6	Possibly contains *A. racemosa* and *A. dahurica*
085	-	**BC043**	-	1.3	5.5	9.2	Possibly *A. racemosa*
086	-	**BC044**	-	ND	ND	ND	There was not enough DNA present to conduct the tests
087	-	**BC045**	-	-1.0	-2.0	1.75	Not able to conclude as C_t values were high and no sequence data

$\Delta\Delta C_t$: $[(C_t \text{ specific} - C_t \text{ generic})_{sample} - (C_t \text{ specific} - C_t \text{ generic})_{reference}]$. A value of 0.0 +/-1.0 is a clear positive result. A value > 6.0 is a clear negative.
Barcode sequence. ITS, the nuclear ribosomal internal transcribed spacer barcode sequence or *maturase K* barcode sequence. The nearest hit by BLAST search is indicated. Chemistry results obtained by Sharaf et al., 2016.

Table 42: Sequencing information for the commercial product DNA extractions – BLAST matches shown

Sample number	Extraction number	Region sequenced	Sequence match	Accession number	Match Percentage	MOTU
AHPA-BC001	1032	ITS	*A. dahurica*	FJ525885.1	97%	*A. dahurica*
AHPA-BC003	1105	*matK*	*A. racemosa*	KU662878.1	94%	N/A
AHPA-BC007	1095	ITS	*A. racemosa*	Z98296.1	94%	*A. racemosa*
AHPA-BC008	1096	*matK*	*A. racemosa*	KU662878.1	99%	N/A
AHPA-BC013	1101	ITS	*A. racemosa*	GQ409509.1	97%	*A. racemosa*
AHPA-BC014	S2	ITS	*A. racemosa*	GQ409511.1	96%	*A. racemosa*
AHPA-BC019	S3	*matK*	*A. racemosa*	KU662878.1	97%	N/A
105	BC025	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
107	BC027	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
081	BC039	ITS	*A. racemosa*	GQ409509.1	99%	*A. racemosa*
082	BC040	ITS	*A. racemosa*	KU724194.1	93%	*A. racemosa*

The chemical analysis that was carried out was by Sharaf et al 2016, and was a system based on using UPLC-QTOF-MS (Ultra performance liquid chromatography-quadrupole orthogonal time of flight-mass spectrometry). Reference samples (*A. racemosa, A. podocarpa, A. rubra, A. pachypoda, A. dahurica, A. cimicifuga, Astilbe biternata* and *Serratula chinensis*) were collected and their chemical profiles characterised using the technique. The chromatograms were converted to ion intensity maps and used to perform principle component analysis. The results of the principle component analysis (PCA) are shown in Figure 50 and include 16 commercial Black Cohosh products (Sharaf et al., 2016).

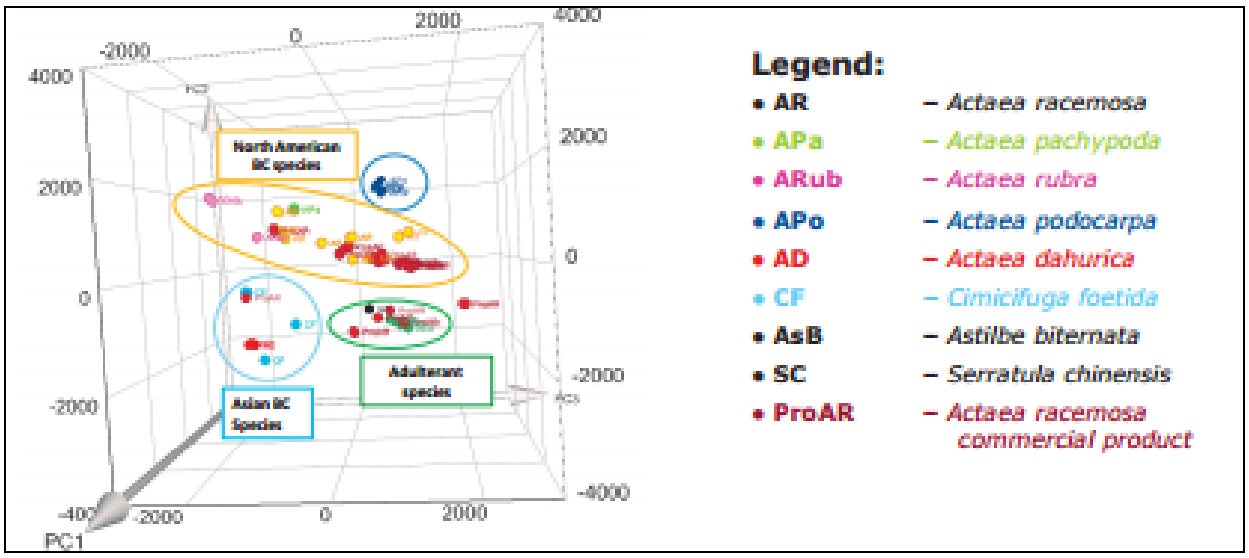


Figure 50: Principle Component Analysis (PCA) of *Actaea* species and adulterant species reference samples and 16 commercial Black Cohosh products (Sharaf et al., 2016)

These results allowed the discrimination of the included species and the results of assigned species for the commercial products are shown in Table 41.

When assigning identification to the tested products using the qPCR assays, several factors came into consideration. If the C_t values of the tests were equally over 35 then the confidence level became weaker. If the $\Delta\Delta C_t$ value exceeded 0.0 +/-1.0 confidence became weaker. If a sample gave a test result exceeding 0.0 +/-1.0 but under 6.0 then sequencing was the next desirable step to provide a confirmation. This was not always possible with this group of samples for a number of reasons. The sequence data was matched to accessions on GenBank by using BLAST. The accessions for ITS and

matK were analysed as described in Chapter 2: Genetic analysis of Black Cohosh, *Actaea racemosa,* and potential adulterant species. These results were purely used to reinforce the qPCR data.

The first of the products in the table, 1032, was shown to be completely substituted with *A. dahurica.* The qPCR gave a positive detection for *A. dahurica* and a negative detection for the other species. The sequencing of the ITS region also confirmed this. Interestingly, the chemistry results also showed substitution by an Asian species, but this was identified as *A. cimicifuga*. The level of certainty for the DNA testing is high as the starting material for the qPCR reference sample was morphologically identified from a DNA bank herbarium sample, the sequenced ITS region matches *A. dahurica* from the GenBank database and the *A. dahurica* accessions are grouped together in the DNA tree. In chapter 2 it was clearly shown that all the *A. dahurica* accessions are clearly distinguished from *A. cimicifuga* and *A. heracleifolia* as well as all the other *Actaea* species.

AHPA-BC002/1093 and AHPA-BC004/1106 were shown to be *A. racemosa* using the chemical methods and the qPCR assay confirms this but the qPCR assay also detected *A. dahurica* presence.

AHPA-BC006/S1 was identified in the chemistry work as *A. dahurica* or *Astilbe biternata* but in the qPCR assays this was identified as *A. racemosa.* Unfortunately it was not possible to sequence the sample. The qPCR assays do not include *Astilbe biternata* and if this sample was *A. racemosa* contaminated with *Astilbe biternata* this would not have been detected with these assays.

AHPA-BC010/1098 was identified as S*erratula chinensis* using the chemical method. In the qPCR assays this was shown to possibly be *A. dahurica.* Unfortunately the DNA was not successfully sequenced so it cannot be known for sure. Again presence of *Serratula chinensis* cannot be detected using the qPCR assay.

AHPA-BC011/1099 was identified as *A. racemosa* using the chemical method but the qPCR assay gives a less confident result for *A. racemosa* and a stronger result towards *A. dahurica* or *A. cimicifuga*, perhaps indicating a mixed sample.

AHPA-BC012/1100 was identified as *A. cimicifuga* using the chemistry method but the qPCR assay is showing *A. racemosa* and *A. dahurica.*

For AHPA-BC015/1107 and AHPA-BC016/1108, they were both identified as *A. racemosa.* The qPCR results show a negative result for *A. dahurica* and *A. cimicifuga* and a possible result for *A. racemosa.* The $\Delta\Delta C_t$ score for the *A. racemosa* test was in the region of 3.5 – 4.0 which would usually require sequencing for confirmation. This was not possible due to the low amount of DNA extracted and possible degradation.

Several of the products were clearly identified and the chemistry results, sequencing results and qPCR assay were in agreement; AHPA-BC003/1105, AHPA-BC007/1095, AHPA-BC008/1096, AHPA-BC013/1101 and AHPA-BC014/S2. This gives further validation to the qPCR assays. All were confirmed to be *A. racemosa.*

The remainder of the samples were collected independently of the AHPA samples and so were not tested chemically. Four of the products were shown to contain *A. racemosa* by the qPCR assay and sequencing; 105/BC025, 107/BC027, 081/BC03 and 082/BC040. Other products that tested positive for *A. racemosa* without sequencing confirmation were 104/BC024, 120/BC030, 121/BC031, 083/BC041 and 085/BC043. The remaining products had a mixture of results, with most testing positive for *A. racemosa* and one of the other species. Two of the products did not have enough DNA present to conclude; 086/BC044 and 084/BC042.

One of the products (121/BC031) was advertised as being polyherbal but only containing *A. racemosa* out of the tested species. It was not possible to sequence this sample of DNA but the qPCR assay did confirm the presence of *A. racemosa.* Some of the samples contained multiple traces when sequencing was attempted, which could indicate the presence of multiple species, i.e. adulteration or sometimes fungal contamination, as the ITS primer pair used can amplify the fungal genome as well as plants. Some samples were simply poor quality in regards to DNA presence i.e. degraded DNA or low yield of DNA.

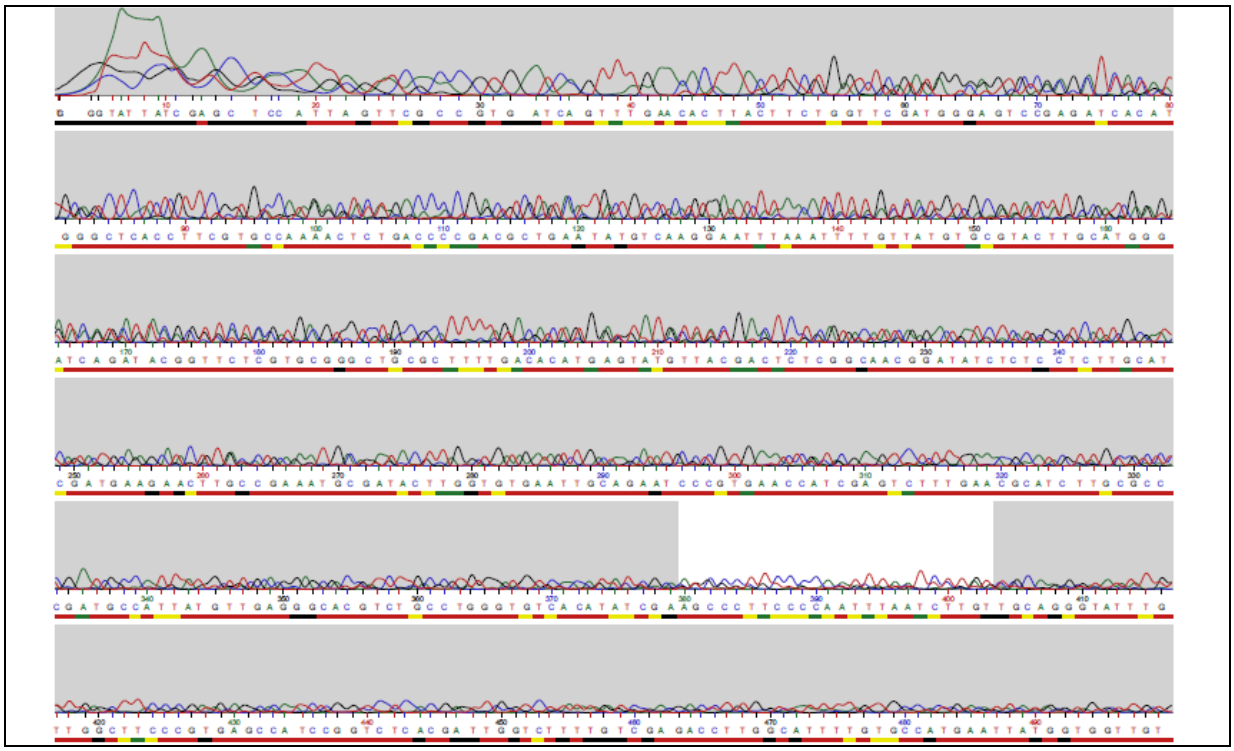


Figure 51: The sequence trace for BC031 highlighting the presence of multiple sequences

4.4 Conclusions and further work

The importance of this chapter was again to develop reliable tests for authentication of Black Cohosh and detection of adulterant species. The utilisation of qPCR as the platform for these assays enables the creation of highly sensitive tests.

The qPCR assays were successfully validated with sequenced reference samples. This gives a high level of confidence to results that are obtained even when sequencing of the test samples cannot be performed.

It has even been shown that commercial products can be identified using this technique. This was not always possible due to limitations with amount and quality of DNA that can be extracted but of 34 commercial samples it was possible to obtain results for 32. A good proportion of these results could be backed up with sequencing, chemical testing or both.

The results of these tests for the assay are very provoking. The addition of the generic primer has turned these assays into a great tool for identification of *Actaea racemosa* and detection of adulterant species. These qPCR assays could also be developed quite quickly for some of the other PlantID species too if it was desirable for those species to be detected. It would also be simple to develop tests for species from other families that are well known to be adulterants of Black Cohosh products. These species will vary a lot more genetically making them easier to detect.

This assay would require little skill to carry out and some simple sums using the C_t values to find a score. Confidence can be put into samples achieving a C_t under 35, if there is low deviation between triplicates and if the scoring is 0.0 +/-1.0. For samples that do not achieve scoring within the confidence range, sequencing can be carried out where possible.

The qPCR assays results agreed with the sequencing results every time where one species was detected. There were some incidences where products/samples were found to be adulterated with other *Actaea* species and also completely substituted. Five of the seventeen UK products were found

to be adulterated. It must be said that these products were only a minute number compared to what is available. There are many batches within each brand and many products available. Within the products provided from the American market there were a few adulterations and even complete substitutions found. From the results it looks as though six or maybe even seven of the products are contaminated with undeclared species. The chemistry tests did not always agree with the DNA based tests and there are several possible explanations for this. One explanation is that there was a misidentification with the reference material used for building up the chemical identification technique (details not known – only final results made available). From the DNA results it looks as though the reference material used to develop the chemistry test was the wrong way round, i.e. the *A. dahurica* material was *A. cimicifuga* and the *A. cimicifuga* material was *A. dahurica*. The origins of the reference materials used for development of the chemistry tests are not known to the author of this thesis. The chemistry test seems to have limitations for detecting contamination of products that contain a mixture of species, which could explain why some of the chemistry results didn't match the qPCR assay results. The strong point of the chemical testing is detecting complete substitutions. Detection of multiple species was not present with the chemical tests so this could explain why the results do not agree; one of the species present could not be detected with the qPCR assay as it was not in the scope and the other species could not be discovered by the chemical test as only the more abundant species was detected. The qPCR assays did not agree with samples that were identified as containing *Astilbe biternata* or *Serratula chinensis* because detection of these species is not possible with these qPCR assays.

This adds to the value of the assay showing that there is a requirement for a more robust technique which DNA testing can offer. The DNA tests are sensitive and can be designed to detect many species, as many as is required for a sample as long as the ability to design and optimise species-specific primers is possible.

5 Investigation into potential hepatotoxicity of three *Actaea* species

5.1 Introduction

As mentioned in the main introduction, the reputation of Black Cohosh has come under jeopardy and the safety of the herbal medicine has been heavily scrutinised. The case reports of liver damage, although not conclusive, have been enough to require a warning label on commercial products in countries across the globe (Mahady et al., 2008).

This chapter aims to answer the questions; why do some individuals who are taking Black Cohosh preparations experience liver injury? Does *Actaea racemosa* cause toxicity to the liver or not? Do Asian *Actaea* species cause toxicity to the liver or not?

5.1.1 Existing investigations into the safety of Black Cohosh

There have been many clinical trials conducted for Black Cohosh, with efficacy and safety in mind, over the years. None of these trials have shown Black Cohosh to cause hepatotoxicity and most of them have good evidence to suggest efficacy of the herb (Nappi, 2005, Osmers et al., 2005, Wuttke et al., 2006, Molla et al., 2011). The numerous isolated cases of liver damage are more of an issue. Although many of these case reports have been reviewed and found to show a poor connection to Black Cohosh consumption, the sheer number still has a damaging effect on the reputability of Black Cohosh use. (Mahady et al., 2009, Teschke et al., 2011, Teschke et al., 2014). There have been extensive trials of Black Cohosh use with rats used as a model. Again these trials have shown Black Cohosh to be safe (Briese et al., 2007, Mazzanti et al., 2008) (Kapur et al., 2010), non-oestrogenic (Liske et al., 2002, Wuttke and Seidlová-Wuttke, 2015) and even protective of bone health (Seidlova-Wuttke et al., 2005) and showing signs of anti-cancer properties (Nisslein and Freudenstein, 2004, Einbond et al., 2008, Yue et al., 2016).

There has not been much research in using human cell models *in vitro* to explore the effect of Black Cohosh on the liver. Huang et al used the MTT assay to assess the effect of three Black Cohosh extracts; 40% isopropanol, 75% and 80% ethanol on a human hepatocyte derived cell line; HepG2. They found that none of the extracts inhibited growth of HepG2 cells significantly therefore demonstrating safety in this experiment ((IC_{50}) ranging from 21.9 µg/ml to 65.0 µg/ml). They proposed that herb-drug interactions rather than direct hepatotoxicity could be the cause of the case reports as the extracts did inhibit CYP isoenzymes (Huang et al., 2010). In response to the proposition that herb-drug interactions are the cause of liver injury in Black Cohosh patients, there have been rodent based models employing gene expression to study pathways. Pang et al treated mice with Black Cohosh extract and could not detect induction of liver specific Cyp3a11. Cyp3a11 was chosen for study as it is the major P450 isoenzyme expressed in the liver and functions in drug metabolism. Liver enzymes used for markers of liver injury were also unaffected (Pang et al., 2011a). The sporadic cases of liver injury in some individuals taking Black Cohosh is still damaging the reputation of the medicinal plant and tests specifically designed to answer the question of whether or not *Actaea racemosa* is toxic to the liver are required.

5.1.2 Possible other causes of hepatotoxicity in adverse case reports of Black Cohosh

There has been speculation that the cases of liver damage could be caused by substitution or even adulteration with closely related Chinese Sheng Ma (translation: ascending hemp) species. These include *A. heracleifolia*, *A. cimicifuga* and *A. dahurica*. Some occasions, where products that have been implicated in cases of hepatotoxicity, have tested positive for adulteration with Chinese *Actaea* (Jiang et al., 2006, Jordan et al., 2010, Masada-Atsumi et al., 2016). In Chinese medicine Sheng Ma is taken as an aqueous decoction i.e. boiled in water. Water versus ethanol as a solvent would render different products and therefore different pharmacological effects on the body.

The point that needs to be addressed is the effect of the Asian *Actaea* species on the liver. Tian et al 2005 and 2007 found that *A. cimicifuga* and *A. dahurica* show cytotoxic activity on HepG2 cells. They

found that there was less effect on primary mouse hepatocytes and they believed that this could mean an anti-tumour property (Tian et al., 2005, Tian et al., 2007). There has been little research into the effect of Chinese *Actaea* species on the liver and no studies specifically designed to assess if they are toxic. This is required to try to answer the question of why some individuals who are taking Black Cohosh experience liver injury.

5.1.3 Approach to assessment of hepatotoxicity in this investigation

In this piece of work the potential hepatotoxicity of two Chinese species, otherwise known as Sheng Ma; *A. cimicifuga* and *A. dahurica*, along with *A. racemosa* was investigated by treating cultured human hepatocyte cell lines with 60% ethanol extracts. The *A. racemosa* material has been identified using morphology, DNA testing and chemical testing. The two Sheng Ma samples that were included; one labelled as *Actaea dahurica* and the other labelled as *Actaea cimicifuga,* were also analysed using DNA and HPTLC testing. The results suggested that both materials were mixed.

The main experiment in this chapter is a large hepatotoxicity related gene expression study. This was designed to measure changes in expression of key genes in hepatocyte derived cells following the exposure to *Actaea* extracts including Black Cohosh and Sheng Ma species. These changes in gene expression can help determine the effect of the extracts on the cells and what disease pathways these are related to if any. A hepatotoxicity pathway related qPCR array from Qiagen was chosen to screen the effects of the extract treatment. The array was made up of 96 tubes, 84 of which are individual gene tests and the remaining 12 being made up of internal controls. Housekeeping genes that are involved in the maintenance of basic cell functions were used to normalise the results of the test genes. The changes of expression in the included genes can be linked to various liver disease pathways including general hepatotoxicity, steatosis, necrosis, nongenotoxic hepatocarcinogenicity, cholestasis and phospholipidosis.

The liver has the important function of uptake, metabolism and excretion of drugs, environmental toxins and xenobiotics. This means that the liver is very susceptible to drug induced liver injury (DILI).

A major function of the liver is to synthesise bile acids. Cholestasis is a condition where the flow of bile fluid from the liver to the duodenum is decreased or completely stopped. If left untreated it can lead to hepatotoxicity. The condition can be caused by many factors including the use of certain drugs (Padda et al., 2011). Steatosis is a condition that if left untreated can progress to the serious disease of cirrhosis. It is characterised by the build-up of free fatty acid and triglyceride esters within hepatocytes. The mechanism of drug induced steatosis is not understood but is thought to be somewhat connected with mitochondria as they play an extensive role in lipid metabolism. Another theory is that the process of excreting and converting free fatty acids to very low density cholesterol is hindered (Rabinowich and Shibolet, 2015). Injury of the liver leads to hepatic necrosis through many different pathways dependant on the cause of the disease so detection of increased necrosis related genes can be useful for studying hepatotoxicity (Guicciardi et al., 2013). Nongenotoxic hepatocarcinogenicity is the ability of a substance to cause cancer to the liver through a pathway not linked to damage of DNA. The pathway in which Nongenotoxic hepatocarcinogenicity occurs is not fully understood but it is thought that changes in oxidative stress, peroxisome proliferation, control of metabolic enzymes, cellular communication and disruption of apoptosis and cell proliferation, are linked from previous studies that have been carried out (Lee et al., 2013). Including genes in the array that are involved in these pathways allows study of the nature of toxicity if present. Phospholipidosis is a condition caused by faults in lysosomes. Lysosomes have the role of metabolising phospholipids and therefore any substance that impairs the pathways associated with this task will result in build-up of phospholipids. A substance can be attributed to causing phospholipidosis by study of lysosomal pathways (Shayman and Abe, 2013). The remaining genes that are to be assessed with the qPCR array are related to other pathways of damage not associated with the previous diseases listed. Many of the genes included in the qPCR array were characterised in a previous study with drug controls known to cause certain liver injuries (Zidek et al., 2007). The gene expression assay approach was chosen as there had not yet been such a wide screening of the effect of Cohosh extracts on so many liver damage associated genes.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and LDH (Lactate dehydrogenase) assays were also used to analyse the effect of the extracts on hepatocyte derived cells.

The MTT assay measures cell viability by assessing the ability of the cells to convert MTT reagent to formazan. Cells lose the ability to metabolise the MTT reagent when they die so it is presumed that the amount of the metabolite formazan that is produced is proportional to the number of viable cells. This test will show if the treatment of the *Actaea* extracts respectively lead to cell death.

The LDH assay was used to assess how much LDH was released by the cells during treatment with *Actaea* extracts. This assay has the ability to measure cytotoxicity as apotosis and necrosis of the cell cause permeation of the cell membrane allowing release of LDH. Therefore the detection of increased levels of LDH in the cell culture medium in controlled conditions can indicate that the test substances cause cytotoxicity.

HepaRG™ cells were chosen for the LDH assay and the gene expression study as cells were required that were as close to normal functioning liver cells as possible. HepaRG™ cells have been shown to be an excellent model for the human liver and retain metabolic activity to a higher level compared to other hepatocyte derived cell lines (Mann et al., 2017). The HepaRG™ cells were terminally differentiated (unable to proliferate and therefore single use due to limited stock), so another cell line was required for the MTT assay. HepG2 cells have also proven to be a good model for toxicity assessment (Saad et al., 2006) and so these were also employed for the LDH and MTT assay post treatment with extracts.

The gene expression study started with a pilot experiment of single arrays for one concentration of extract along with a positive control. The positive control chosen was paracetamol (APAP, Acetaminophen) as it has been proven to be toxic to the liver (Jeong et al., 2006). The main experiment concentrated on comparing the Black Cohosh extract and Sheng Ma extracts at three different concentrations and was conducted with biological triplicates each analysed with a separate

array. The paracetamol control was utilised also. The LDH assay was utilised for assessment of treatment on both included cell lines and the MTT assay was used to assess the treatment of extracts on the HepG2 cells.

5.1.4 Aims:

- To produce industry standard extracts from plant material of known identity - the identity to be verified by HPTLC, DNA and morphological assessment.
- To use the manufactured 60% ethanol extracts to treat hepatocyte cell lines in vitro and measure effects using qPCR gene expression arrays, an LDH assay and a MTT assay. This will provide a much needed direct comparison of Black Cohosh and Sheng Ma with relation to hepatotoxicity.

5.2 Materials and method

5.2.1 Preparation of 60% ethanol extracts

As the majority of commercially available products contain 60% ethanol extracts, it was decided to follow suit and create the extracts to be used in this chapter with the same method. Sheng Ma, when used in Chinese medicine, is administered as an aqueous decoction. However if any Sheng Ma species were used to manufacture products labelled as 'Black Cohosh Extract', they would most likely be subjected to alcoholic extraction. For this reason ethanol extracts of the Sheng Ma material were performed to reflect what would happen in the case of substitution or adulteration of Black Cohosh products with Sheng Ma species.

To begin with the plant materials were milled to a fine powder and 100 g was placed in a round bottomed flask with 700 ml of 60% ethanol. The mixture was attached to a rotary evaporator (Buchi, Essen, Germany) without vacuum at this stage. The round bottomed flask was set to rotate in a water bath at 60°C for 60 minutes. After the 60 minute incubation was over the slurry of plant material was filtered using a Buchner funnel and T100 filter under vacuum. Once completely dry the left over solid

material was placed back into the round bottomed flask with 700 ml of 60% ethanol and incubated again in the 60°C water bath for 60 minutes. After the 60 minute period the slurry was again filtered. The two wet extracts were combined and dried using a rotary evaporator under vacuum. The Sheng Ma extracts, outlined in Table 43, were very difficult to dry and were finished off using freeze drying. This involved freezing the wet extract rapidly in a methanol bath and attaching to a freeze dryer under vacuum which removed the water using sublimation. The resulting dry extracts were removed from the flasks and passed through a sieve to reduce to a powder.

All information for the plant materials used in this chapter can be found below in Table 43:

Table 43: The material used for 60% ethanol extract manufacture and resulting extract information

Sample label	Common name	Sample number	Sample type	Source
Actaea racemosa	Black Cohosh	DR10-014-A	Root and rhizome	Staffort, DHU, Germany
Actaea cimicifuga	Sheng Ma	Ch.B.13884K116-A	Sliced rhizome	SynoPhyto, China
Actaea dahurica	Sheng Ma	Ch.B.01230952	Sliced rhizome	SynoPhyto, China

5.2.2 Analysis of 60% ethanol extracts

5.2.2.1 High Performance Thin Layer Chromatography (HPTLC)

The fingerprint of each extract was analysed using HPTLC in order to attempt identification. For this technique a semi-automated HPTLC system was used (CAMAG, Switzerland). This included an automated plating machine, an automated development tank, a hot plate and equipment capable of imaging under several UV wavelengths and white light.

The process began with weighing out 200 mg of extract, placing in 10 ml of methanol and sonicating until visually dissolved. For the reference standards 1 mg was weighed out and dissolved in 10 ml of methanol. The included reference standards were actein, 23-epi-26-Deoxyactein, caffeic acid, chlorogenic acid and Isoferulic acid. Unfortunately cimicifugin and norcimicifugin were unavailable at the time of experimentation so these could not be included. The solutions were placed in labelled

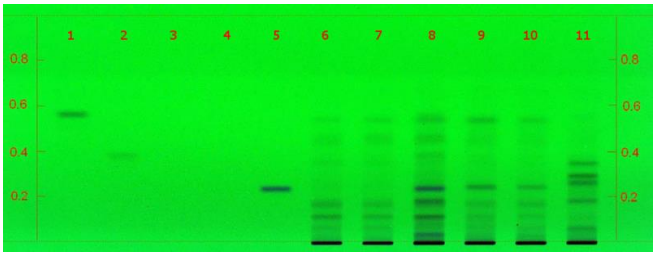
glass bottles and placed in the rack of the plating equipment. A silica gel 60 F254 plate (Merck, Kenilworth, New Jersey, United States) was selected and clipped into position on the plating equipment. Using the machine software the plate layout was designed to maximise use. This resulted in 8 mm bands using 2 µL of sample.

Three methods of analysis were used which were each developed by CAMAG Laboratories, Switzerland.

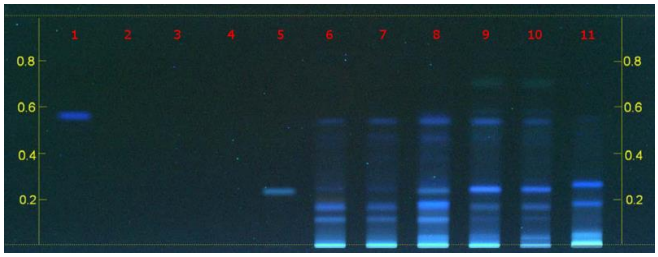
5.2.2.1.1 A CAMAG method for identification of Black Cohosh and possible contaminants.

The mobile phase was made up of toluol, ethyl formate and formic acid at a ratio of 50:30:20. Derivitisation was performed using a sulphuric acid reagent (20 mL of sulphuric acid added to 180 mL of ice cooled methanol) which was sprayed evenly on the plate and then dried at 100°C for five minutes. The plate should be imaged at UV at 366 nm prior to derivitisation and then after derivitisation imaging under white light and UV at 366 nm.

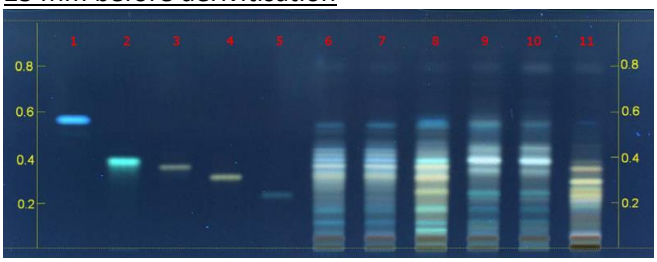
Example plates are shown in Figure 52 which was adapted from the CAMAG protocol.



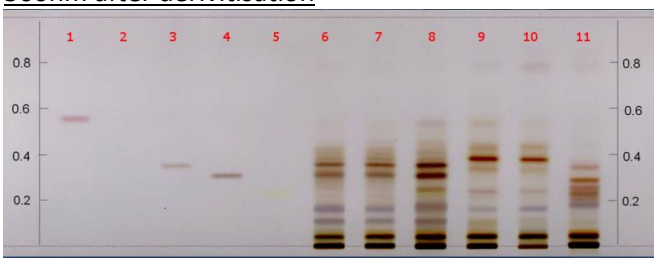
254nm before derivitisation



366nm before derivitisation



366nm after derivitisation



White light after derivitisation

1. 2 µL Isoferulic acid
2. 2 µL Norcimifugin
3. 2 µL Actein
4. 2 µL 23-epi-26-Deoxyactein
5. 2 µL Cimifugin
6. 2 µL *A. racemosa*
7. 2 µL *A. racemosa*
8. 2 µL *A. cimicifuga*
9. 2 µL *A. heracleifolia*
10. 2 µL *A. dahurica*
11. 2 µL *A. podocarpa*

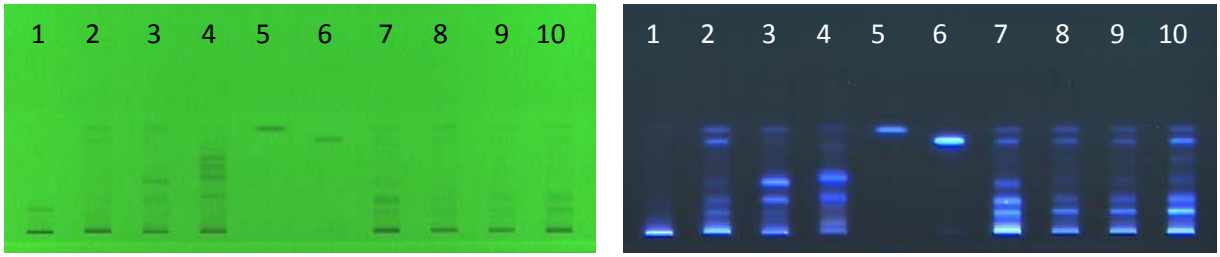
Figure 52: Example HPTLC plates for analysis of *A. racemosa* and adulterant species using a combined method for detection of plant acids and triterpene glycosides – figure adapted from CAMAG protocol (CAMAG)

The reference bands should be cross checked for presence in the test samples. Unfortunately cimicifugin and norcimicifugin were not available when conducting this experiment so the example plates will be used to determine the position of where these bands could be on the test plates.

5.2.2.1.2 The CAMAG plant acids detection method.

The mobile phase was made up of toluol, ethyl formate and formic acid at a ratio of 50:30:20. The plate was first heated to 100°C for three minutes. Derivitisation was performed using a diphenylborinic acid reagent (1.0 g of diphenylborinic acid dissolved in 200 mL ethyl acetate), which was sprayed evenly on the plate and then dried in a stream of cool air. Imaging is recommended under UV light at 366 nm.

Example plates are shown in Figure 53 which was adapted from the CAMAG protocol.



254nm before derivitisation

366nm after derivitisation

1. *Serratula* sp. (adulterant)
2. *Actaea racemosa*
3. Sheng Ma (adulterant, *A. dahurica*)
4. *Actaea podocarpa*
5. Isoferulic acid
6. Caffeic acid
7. *Actaea racemosa*
8. *Actaea racemosa*
9. *Actaea racemosa*
10. *Actaea racemosa*

Figure 53: Example finished HPTLC plates for identification of Black Cohosh using a plant acids based method – figure adapted from a CAMAG protocol (CAMAG)

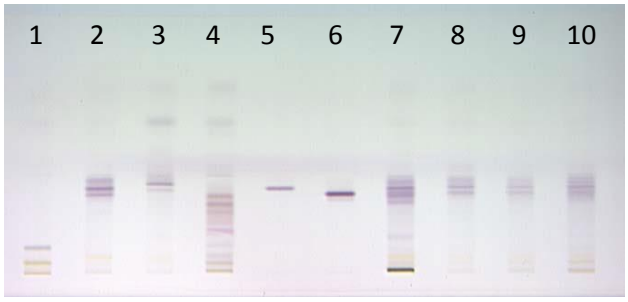
The presence of the reference bands in the test samples will help to determine which species is present.

5.2.2.1.3 The CAMAG triterpenes method.

The mobile phase was made up of toluol, ethyl formate and formic acid at a ratio of 50:30:20. Derivitisation was performed using a sulphuric acid/anisaldehyde reagent (10 mL sulphuric acid added to 170 mL of ice cooled methanol, 20 mL of acetic acid and 1 mL of anisaldehyde), which was

sprayed evenly on the plate and then dried for 5 minutes at 100°C. Imaging under white light is recommended.

Example plates are shown in Figure 54 which was adapted from the CAMAG protocol.



White light after derivitisation

1 *Serratula* sp. (adulterant)
2 *Actaea racemosa*
3 Sheng Ma (*A. dahurica*)
4 *Actaea podocarpa*
5 Actein
6 23-epi-26-Deoxyactein
7 *Actaea racemosa*
8 *Actaea racemosa*
9 *Actaea racemosa*
10 *Actaea racemosa*

Figure 54: Example finished HPTLC plate for identification of Black Cohosh using a triterpene glycosides based method – figure adapted from a CAMAG protocol (CAMAG)

The presence of 23-epi-26-Deoxyactein is used to determine if the species is *Actaea racemosa.* Although it is not shown in the example plate, Actein is present in *Actaea cimicifuga* as well as *Actaea racemosa.*

5.2.2.2 DNA based analysis of the materials used to manufacture 60% ethanol extracts

The samples outlined in Table 43 were subjected to DNA extraction following the method outlined in section 2.2.2 Extraction of DNA from plant material using the Qiagen DNeasy Mini Plant Kit. The resulting DNA extracts were then amplified using PCR as described in 2.2.5 Standard (End-point) Polymerase Chain Reaction with ITS1 and ITS4 primers. The ITS PCR products were sent to an external supplier and sequenced where possible. The DNA extractions were also analysed using the qPCR assays developed in Chapter 4 Development of a qPCR assay for the authentication of *A. racemosa* and detection of potential adulterants, which included a test for *A. racemosa, A. dahurica* and *A. cimicifuga.*

5.2.3 Culture of HepaRG™ cells - Initial thawing, counting and plating of cells

All cell culture work was carried out in an appropriate sterile laminar flow hood using aseptic techniques and conditions. The HepaRG™ Thaw, Seed and General purpose medium (Biopredic, Saint-Grégoire, France) was pre-warmed in a 37°C water bath. Before use of the medium could occur, the outside of the bottle had to be cleaned with 70% ethanol to maintain sterility inside the laminar flow hood. Once the bottle was clean, 9mL of medium was placed into a sterile 40 mL polystyrene round bottomed container. The cells were placed into liquid nitrogen for cryopreservation upon arrival to the lab, so the next step was to thaw the HepaRG™ cells (Biopredic, Saint-Grégoire, France). The cryovial of cells was removed from liquid nitrogen storage. Inside the flow hood the lid of the cryovial was opened slightly to release any internal pressure and then closed again. The vial was placed into the 37°C water bath for two minutes, agitating throughout, without submersion. Again, to maintain sterility, the outside of the cryovial was cleaned with 70% ethanol before placing under the laminar flow hood. The thawed cells were transferred to the tube containing the 9mL of warmed medium resulting in a 1:10 ratio. The inside of the cryovial was rinsed once with 1mL of cell suspension and then returned back to the rest of the suspension. The suspension was then centrifuged for 3 minutes at 500 x *g* at room temperature. The medium supernatant was removed and the cells resuspended in 5mL of fresh medium.

Cell counting and viability assessment was achieved using Tryptan Blue staining and a counting chamber. The Tryptan Blue stock solution (Sigma-Aldrich, St. Louis, Missouri, United States) was at a concentration of 0.4% in PBS buffer. The required concentration was 0.05% and so was appropriately diluted. The counting chamber was then prepared by first cleaning it with lens paper and then placing a cover slip over the counting grid. To a tube, 400 µL of 0.05% Tryptan Blue was placed with 100 µL of mixed cell suspension. The solution was then transferred to the counting chamber. The live cells do not take up the dye and remain clear and the dead cells are stained blue. Live and dead cells were counted and the numbers noted. The cell concentration (million cells/mL) and viable cell number was calculated. The percentage of viable cells is used to gain an exact number of viable cells.

For the experiments carried out in this work 24 well plates were used. The recommended number of viable cells per well for a 24 well plate was 0.48×10^6, so the appropriate amount of cells was added to each well with a measure of medium to ensure an overall volume of 500 µL was placed in each well.

For a toxicity study the cells need to culture for 7 days prior to incubation with the test substance. The schedule in Table 44 was followed.

Table 44: A table outlining key activities from culturing to treatment of HepaRG™ Cells

Day	Day of the week	Activity	Photomicrograph?
0	Thursday	Thaw and seed the cells	
1	Friday	Renew the medium	Take a photomicrograph
4	Monday	Renew the medium	Take a photomicrograph
6	Wednesday	Renew the medium	Take a photomicrograph
7	Thursday	Renew the medium and incubate with the test substance for 24 hrs	
8	Friday	Remove medium and begin RNA extraction	

On the 7th day of culturing, the cells were treated with the following extracts and preparations;

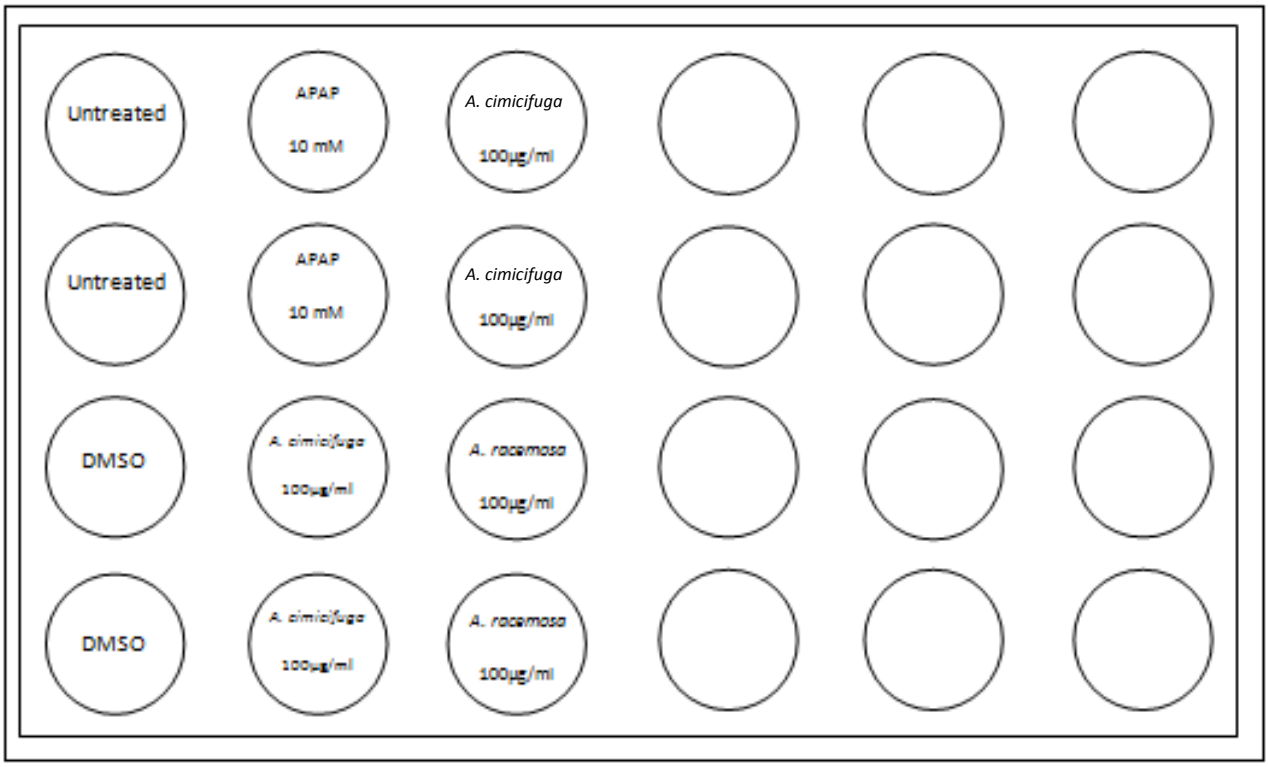


Figure 55: The layout of the planned pilot experiment – 24 well plate

APAP (acetaminophen, paracetamol) was used as a control for toxicity as it has been shown to induce various toxicity pathways in cultured liver cells.

5.2.4 Culture of HepG2 cells

Aseptic technique was adhered to as with any cell culture experimentation. Minimum Essential Eagle's Medium supplemented with 10 mM L-Glutamine, 1% Non-Essential Amino Acid solution, 5% foetal calf serum and 1% Pen-Strep solution (ThermoFisher Scientific, Waltham, Massachusetts, United States), was used for culture of HepG2 cells. The HepG2 cells, unlike the HepaRG™ cells are an immortalised adherent cell line that continued to grown and divide. For these reason they had to be passaged or divided 1:3 every 2-3 days once thawed and seeded to a T75 flask. In order to achieve the cells were removed from the flask once confluency reached 90% (usually within 2-3 days) with trypsin. The flasks of cells were maintained in an incubator at 37°C with 5% CO_2. Once a stable cell line was in place, the cells were applied to a 24 well plate and grown to 90% confluency prior to

treatment with extracts for the LDH assay and to a 96 well plate for treatment with extracts for the MTT assay.

5.2.5 RNA extraction using Qiagen RNeasy mini Kit

RNA extraction was achieved using the Qiagen RNeasy® Mini Kit (Qiagen Inc.,CA). The protocol for "Animal Cells Spin" was adhered to as follows;

The culture medium was completely removed and a phosphate buffered saline (PBS) (ThermoFisher Scientific, Waltham, Massachusetts, United States) wash performed once to ensure complete removal. The PBS was then removed. The number of cells present in each well was $<5 \times 10^6$ so 350 µL of lysis buffer RLT was applied to each well. The lysate was collected using a pipette and placed into a microcentrifuge tube and vortexed. The next step was to homogenise the cells and the method of choice was to use a QIAshredder spin column. The lysate was pipetted directly to the QIAshredder spin column and centrifuged for 2 minutes at full speed. To the homogenised material, 350 µL of 70% ethanol was added and mixed by pipetting. The sample (700 µL) was transferred to an RNeasy spin column; the lid was gently closed and centrifuged for 15 seconds at ≥8000 × *g* to wash the spin column membrane. The flow through was discarded. DNA elimination was very important for the procedures that used the extracted RNA so optional on column DNA elimination was carried out. To the RNeasy spin column, 350 µL buffer RW1 was added. The lid was gently closed and centrifuged for 15 seconds at ≥8000 × *g* to wash the spin column membrane. The flow through was discarded. For each sample a mixture of 10 µL of DNase 1 stock solution and 70 µL of buffer RDD was prepared, the solution was mixed gently by inversion of the tube and brief centrifugation to ensure residual liquid from the sides of the tube was incorporated. The resulting solution (80 µL) was added directly to the RNeasy column membrane and incubated at room temperature for 15 minutes. After incubation, 350 µL of buffer RW1 was added to the RNeasy spin column. The lid was gently closed and centrifuged for 15 seconds at ≥8000 × *g* to wash the spin column membrane. The flow through was discarded. To the RNeasy spin column, 500 µL of buffer RPE was added. The lid was gently closed and centrifuged for

15 seconds at ≥8000 × *g* to wash the spin column membrane. The flow through was discarded. A further 500 µL of buffer RPE was added to the RNeasy spin column. The lid was gently closed and centrifuged for 2 minutes at ≥8000 × *g* to wash the spin column membrane. The flow through was discarded. For an extra measure, the Rneasy spin column was placed into a new collection tube, and centrifuged at full speed for 2 minutes to ensure complete removal of buffer RPE. The RNeasy spin column was placed into a new collection tube and 50 µL of RNase free water was added. A final centrifugation step was carried out.

The resulting RNA was quantified using a spectrophotometer.

5.2.6 Analysis of extracted RNA using BioRad Experion RNA Standard Sensitivity Chip

The first step was to equilibrate the kit reagents to room temperature; this included the Loading Buffer, Stain and Gel (Biorad, Hercules, California, United States). The ladder element of the kit was thawed on ice. The kit reagents were briefly centrifuged and the ladder was immediately returned to ice. The electrodes of the Experion system (Biorad, Hercules, California, United States) were cleaned using the cleaning chip loaded with Experion Electrode Cleaner (Biorad, Hercules, California, United States). They were then cleaned twice using the DEPC cleaning chip loaded with DEPC treated water. For the gel preparation 600µL of Gel was added to the supplied filter tube and centrifuged at 1500 x *g* for 10 minutes. To prepare the Gel Stain, 65 µL of Gel was added to a separate sterile tube with 1 µL of Stain, vortexed and centrifuged briefly.

To the yellow GS well on the chip, 9 µL of Gel Stain was carefully applied. On the Priming Station the programme B1 was selected as shown on the chip and the chip was carefully put in place. The programme was started and the once finished the chip was removed. The chip was visually inspected for any signs of air bubbles as these have a detrimental effect to the results.

The ladder and test samples were prepared by adding 2 µL respectively into separate labelled tubes and treated for 2 minutes at 70°C. The tubes were centrifuged briefly afterwards and returned to ice.

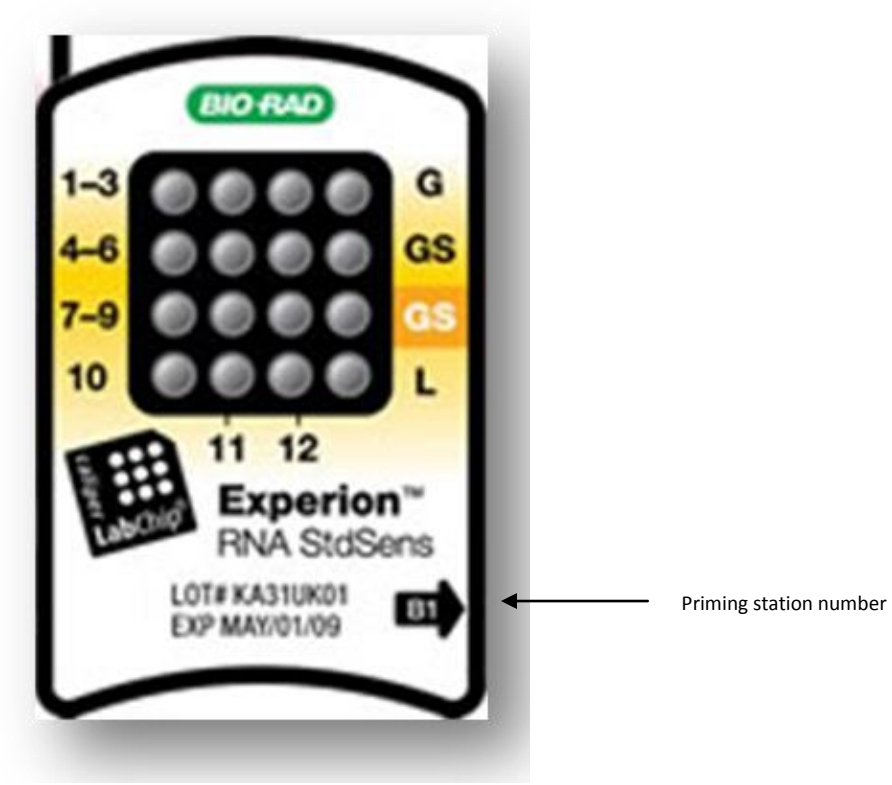


Figure 56: An example of the layout of a standard sensitivity Experion™ RNA Chip

To well L, 1 µL of Ladder was carefully applied. To well G, 9 µL of filtered Gel was applied. To the remaining GS well, 9 µL of Gel Stain was applied. To wells 1-12 and well L 5 µL of loading buffer was applied. Finally 1 µL of denatured sample was added to each of wells 1-12. The chip was placed into the Experion system and run using the appropriate programme. Results were given at the end.

5.2.7 cDNA synthesis using the RT^2 First Strand Kit

The reagents of the RT^2 First Strand Kit (Qiagen Inc.,CA) were first thawed out and then briefly centrifuged. The genomic DNA elimination mix was prepared according to Table 45. To begin with 4 µL of RNA was used as this was the recommended amount in the manual for a very similar cell line. In later experiments the amount of RNA was quantified and volumes adjusted so that 0.5 µg was present as starting template for the cDNA synthesis reaction.

Table 45: A table to demonstrate the components and amounts needed for genomic DNA elimination

Component	Amount for 1 reaction (µL)

RNA	4
Buffer GE	2
RNase-free water	4
Total	10

The genomic elimination mix was then incubated for 5 minutes at 42°C then placed on ice for 2 minutes. The next step was to prepare the reverse-transcription mix according to Table 46.

Table 46: A table to demonstrate components and amounts required for reverse transcription

Component	Amount for 1 reaction (µL)
5 × Buffer BC3	4
Control P2	1
RE3 Reverse Transcriptase Mix	2
RNase-free water	3
Total volume	10

If more than one reaction was being conducted then each component was added to the required volume. To each completed DNA elimination mix, 10µL of reverse-transcription mix was added and mixed gently with pipetting. The reactions were then incubated for 15 minutes at 42°C followed by a stop temperature of 95°C for 5 minutes. Each reaction was then diluted with 91µL of RNase-free water. The reactions were then immediately stored at -20°C or placed on ice if qPCR was to be carried out.

5.2.8 Preparation and analysis of Quantitative Polymerase Chain Reaction arrays

The RT^2 SYBR Green Mastermix (Qiagen Inc.,CA) was briefly centrifuged to bring all the contents together. The components mix was prepared in a 5mL tube as follows in Table 47.

Table 47: The components and amounts required for application to one Hepatotoxicity RT2 profiler qPCR array

Component	Amount for one array (µL)
2 × RT^2 SYBR Green Mastermix	1350
cDNA synthesis reaction	102
RNase-free water	1248
Total	2700

For analysis of the cDNA the human hepatotoxicity RT^2 profiler array PAHS-093Z was used (Qiagen Inc.,CA). In each well of the plate, 24 µL of the components mix was applied. The plate was checked for the presence of bubbles and then placed into the Icyler (Biorad, Hercules, California, United States). The following programme was used outlined in Table 48.

Table 48: Cycling conditions for running a qPCR reaction and melt curve analysis for each RT^2 profiler array

HotStart DNA *taq* polymerase activation 1 cycle	40 cycles			Melt curve		
	Denature	**Anneal**	**Plate read**	**Start temperature**	**End temperature**	**Rate** (Plate read each incremental increase)
95°C	95°C	60°C		65°C	95°C	0.5°C/Min
10 minutes	15 seconds	1 minute				

Once qPCR was complete the threshold was manually determined using the log view, by selecting an area above the background signal and within the lower one third to one half of the log phase on the plot, also ensuring that the C_t values of the three positive PCR controls in wells H10-H12 were maintained at 20+/-2. This threshold was maintained on all arrays in the same experimental set.

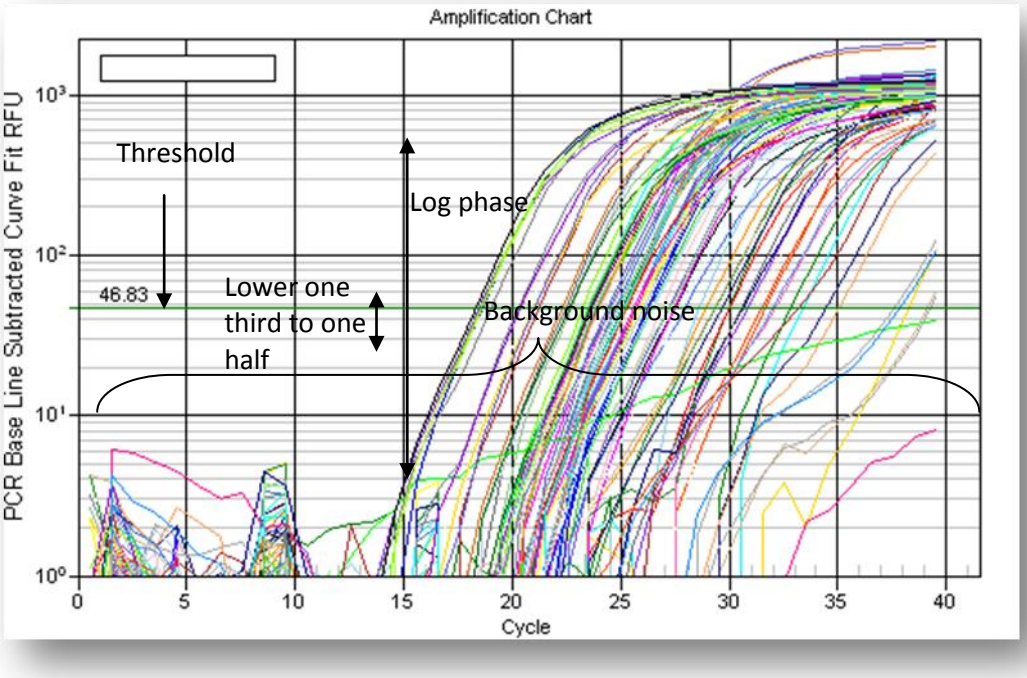


Figure 57: An example of an adjustment of the threshold position on the primary RT2 profiler array – background noise and log phase used to determine position of the threshold

The resulting C_t values were exported to an Excel spread sheet and analysed using the PCR Array Data Analysis Web Based Software (Qiagen Inc.,CA). The software automatically calculates all of the gene expression figures. This involved firstly normalising the data with housekeeping genes. The arithmetic mean of the housekeeping gene panel was used for this purpose. These are outlined on Table 49. In order to achieve normalisation the ΔC_t of each reaction is calculated as follows:

$\Delta C_t = C_t$ (gene of interest) - AVG C_t (chosen housekeeper genes)

Then the quality control data is assessed. The C_t value of the genomic DNA control should be in excess of 35 although this figure can be decided by the researcher, in this case 35 was used. The qPCR array reproducibility test is calculated by taking the average of the three positive PCR control (PPC) tests and the figure should be 20+/-2. The final quality check is the reverse transcription reaction. This is calculated using the positive PCR control tests and the reverse transcription control (RTC). This is calculated by averaging the C_t values of three reverse transcription controls and

subtracting the average of the three positive PCR controls. This figure should not exceed 5. The calculation is as follows:

ΔC_t = (AVG RTC - AVG PPC)

Table 49: A summary of the controls used for each qPCR array – housekeeping genes, genomic DNA detection, reverse transcription control and positive control for PCR.

Well No.	Location	Symbol	Description	Gene name
85	H1	ACTB	Actin, beta	PS1TP5BP1
86	H2	B2M	Beta-2-microglobulin	-
87	H3	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, MGC88685
88	H4	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT
89	H5	RPLP0	Ribosomal protein, large, P0	L10E, LP0, MGC111226, MGC88175, P0, PRLP0, RPP0
90	H6	HGDC	Human Genomic DNA Contamination	HIGX1A
91 – 93	H7 – H9	RTC	Reverse Transcription Control	RTC
94 – 96	H10 – H12	PPC	Positive PCR Control	PPC

After all the steps are complete an overview of the data is given. The next step is to calculate: (2^(- Δ C_t)) for each of the genes of interest. The figures in the control set are directly compared to calculate a fold change:

Fold change ((2^(- ΔΔ C_t)) = ((2^(- Δ C_t)) test sample) / ((2^(- Δ C_t)) control sample)

Fold change values greater than 1 indicate an up regulation or increased expression of a gene and values less than 1 indicate a down regulation or decreased expression of a gene. The fold regulation is the final calculation in terms of gene expression and displays the fold changes in a biologically meaningful way. If a value is greater than 1 it stays the same. If a fold change value is less than 1 the fold regulation is the negative inverse of that value. If at least three biological replicates are included then a student t-test is performed and the value given as a 'P value' which should be at least 0.05. Descriptions of the genes that are included in the qPCR array that was used are shown in Table 50.

Table 50: Positioning and information of the genes analysed

Well No.	Location	Symbol	Description	Gene name	Disease pathway involvement
1	A1	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABC20, CD243, CLCS, GP170, MDR1, MGC163296, P-GP, PGY1	Cholestasis, phospholipidosis
2	A2	ABCB11	ATP-binding cassette, sub-family B (MDR/TAP), member 11	ABC16, BRIC2, BSEP, PFIC-2, PFIC2, PGY4, SPGP	Hepatotoxicity
3	A3	ABCB4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	ABC21, GBD1, MDR2, MDR2, 3, MDR3, PFIC-3, PGY3	Cholestasis
4	A4	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	ABC30, CMOAT, DJS, KIAA1010, MRP2, cMRP	Cholestasis
5	A5	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABC31, DKFZp686E22157, EST90757, MLP2, MOAT-D, MRP3, cMOAT2	Cholestasis
6	A6	ALDOA	Aldolase A, fructose-bisphosphate	ALDA, GSD12, MGC10942, MGC17716, MGC17767	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
7	A7	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	APE, APE1, APEN, APEX, APX, HAP1, REF1	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
8	A8	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	AC, ACDase, ASAH, FLJ21558, FLJ22079, PHP, PHP32	Phospholipidosis
9	A9	ATP8B1	ATPase, aminophospholipid transporter, class I, type 8B, member 1	ATPIC, BRIC, FIC1, PFIC, PFIC1	Cholestasis
10	A10	AVPR1A	Arginine vasopressin receptor 1A	AVPR1	Hepatotoxicity
11	A11	BHMT	Betaine--homocysteine S-methyltransferase	BHMT1	Hepatotoxicity
12	A12	BTG2	BTG family, member 2	MGC126063, MGC126064, PC3, TIS21	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
13	B1	CA3	Carbonic anhydrase III, muscle specific	CAIII, Car3, FLJ36434	Hepatotoxicity
14	B2	CASP3	Caspase 3, apoptosis-related cysteine peptidase	CPP32, CPP32B, SCA-1	Hepatotoxicity
15	B3	CCNG1	Cyclin G1	CCNG	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
16	B4	CD36	CD36 molecule (thrombospondin receptor)	CHDS7, FAT, GP3B, GP4, GPIV, PASIV, SCARB3	Steatosis

17	B5	CD68	CD68 molecule	DKFZp686M18236, GP110, SCARD1	Necrosis
18	B6	CDC14B	CDC14 cell division cycle 14 homolog B (S. cerevisiae)	CDC14B3, Cdc14B1, Cdc14B2, hCDC14B	Necrosis
19	B7	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CAP20, CDKN1, CIP1, MDA-6, P21, SDI1, WAF1, p21CIP1	Nongenotoxic Hepatocarcinogenicity, Necrosis
20	B8	COL4A1	Collagen, type IV, alpha 1	arresten	Necrosis
21	B9	CRYL1	Crystallin, lambda 1	GDH, MGC149525, MGC149526, lambda-CRY	Hepatotoxicity
22	B10	CXCL12	Chemokine (C-X-C motif) ligand 12	IRH, PBSF, SCYB12, SDF1, SDF1A, SDF1B, TLSF, TPAR1	Hepatotoxicity
23	B11	CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	CP12, P3-450, P450(PA)	Hepatotoxicity
24	B12	DDIT4L	DNA-damage-inducible transcript 4-like	REDD2, Rtp801L	Hepatotoxicity
25	C1	DDX39A	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A	BAT1, BAT1L, DDX39, DDXL, MGC18203, MGC8417, URH49	Nongenotoxic Hepatocarcinogenicity, Necrosis
26	C2	DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	ABBP-2, ABBP2, DJ9, EDJ, ERdj3, ERj3, HEDJ, PRO1080, UNQ537, hDj9	Hepatotoxicity
27	C3	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	FLJ21288, HP58, P58, P58IPK, PRKRI	Hepatotoxicity
28	C4	FABP1	Fatty acid binding protein 1, liver	FABPL, L-FABP	Phospholipidosis
29	C5	FADS1	Fatty acid desaturase 1	D5D, FADS6, FADSD5, FLJ38956, FLJ90273, LLCDL1, TU12	Hepatotoxicity
30	C6	FAM158A	Family with sequence similarity 158, member A	C14orf122	Necrosis
31	C7	FASN	Fatty acid synthase	FAS, MGC14367, MGC15706, OA-519, SDR27X1	Steatosis
32	C8	FMO1	Flavin containing monooxygenase 1	-	Hepatotoxicity
33	C9	FXC1	Fracture callus 1 homolog (rat)	TIM10B, TIMM10B, Tim9b	Hepatotoxicity, phospholipidosis
34	C10	GADD45A	Growth arrest and DNA-damage-inducible, alpha	DDIT1, GADD45	Hepatotoxicity
35	C11	GCLC	Glutamate-cysteine ligase, catalytic subunit	GCL, GCS, GLCL, GLCLC	Hepatotoxicity
36	C12	GSR	Glutathione reductase	MGC78522	Hepatotoxicity
37	D1	HAO2	Hydroxyacid oxidase 2 (long chain)	GIG16, HAOX2	Hepatotoxicity
38	D2	HMOX1	Heme oxygenase (decycling) 1	HO-1, HSP32, bK286B10	Hepatotoxicity

39	D3	HPN	Hepsin	TMPRSS1	Phospholipidosis
40	D4	HYOU1	Hypoxia up-regulated 1	DKFZp686N08236, FLJ94899, FLJ97572, Grp170, HSP12A, ORP150	Hepatotoxicity
41	D5	ICAM1	Intercellular adhesion molecule 1	BB2, CD54, P3.58	Cholestasis
42	D6	IGFALS	Insulin-like growth factor binding protein, acid labile subunit	ALS	Hepatotoxicity
43	D7	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	CD130, CDW130, DKFZp564F053, GP130, IL-6RB	Necrosis
44	D8	IPO4	Importin 4	FLJ23338, Imp4, MGC131665	Necrosis
45	D9	KIAA1370	KIAA1370	FLJ10980, MGC126494, MGC126495	Necrosis
46	D10	KRT18	Keratin 18	CYK18, K18	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
47	D11	KRT8	Keratin 8	CARD2, CK8, CYK8, K2C8, K8, KO	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
48	D12	L2HGDH	L-2-hydroxyglutarate dehydrogenase	C14orf160, DURANIN, FLJ12618	Necrosis
49	E1	LGR5	Leucine-rich repeat containing G protein-coupled receptor 5	FEX, GPR49, GPR67, GRP49, HG38, MGC117008	Necrosis
50	E2	LPL	Lipoprotein lipase	HDLCQ11, LIPD	Steatosis
51	E3	LSS	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	FLJ25486, FLJ35015, FLJ39450, FLJ46393, OSC	Phospholipidosis
52	E4	MAOB	Monoamine oxidase B	MGC26382	Hepatotoxicity
53	E5	MAP3K6	Mitogen-activated protein kinase kinase kinase 6	ASK2, MAPKKK6, MEKK6, MGC125653, MGC20114	Necrosis
54	E6	MBL2	Mannose-binding lectin (protein C) 2, soluble	COLEC1, HSMBPC, MBL, MBP, MBP-C, MBP1, MGC116832, MGC116833	Hepatotoxicity
55	E7	MCM10	Minichromosome maintenance complex component 10	CNA43, DNA43, MGC126776	Necrosis
56	E8	MLXIPL	MLX interacting protein-like	CHREBP, MIO, MONDOB, WBSCR14, WS-bHLH, bHLHd14	Necrosis
57	E9	MRPS18B	Mitochondrial ribosomal protein S18B	C6orf14, DKFZp564H0223, HSPC183, HumanS18a, MRP-S18-2, MRPS18-2, PTD017, S18amt	Phospholipidosis, Nongenotoxic Hepatocarcinogenicity
58	E10	NQO1	NAD(P)H dehydrogenase, quinone 1	DHQU, DIA4, DTD, NMOR1, NMORI, QR1	Hepatotoxicity
59	E11	NUS1	Nuclear undecaprenyl pyrophosphate	C6orf68, MGC117249, MGC7199,	Necrosis

			synthase 1 homolog (S. cerevisiae)	MGC:7199, NgBR	
60	E12	OSMR	Oncostatin M receptor	MGC150626, MGC150627, MGC75127, OSMRB	Necrosis
61	F1	SLC51A	Organic solute transporter alpha	MGC39807, OSTA	Cholestasis
62	F2	PDYN	Prodynorphin	ADCA, MGC26418, PENKB, SCA23	Cholestasis
63	F3	PLA2G12A	Phospholipase A2, group XIIA	GXII, PLA2G12, ROSSY	Hepatotoxicity
64	F4	PPARA	Peroxisome proliferator-activated receptor alpha	MGC2237, MGC2452, NR1C1, PPAR, PPARalpha, hPPAR	Steatosis
65	F5	PSME3	Proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	Ki, PA28-gamma, PA28G, REG-GAMMA	Necrosis
66	F6	PYGL	Phosphorylase, glycogen, liver	GSD6	Hepatotoxicity
67	F7	RB1	Retinoblastoma 1	OSRC, RB, p105-Rb, pRb, pp110	Hepatotoxicity
68	F8	RDX	Radixin	DFNB24	Cholestasis
69	F9	RHBG	Rh family, B glycoprotein (gene/pseudogene)	SLC42A2	Necrosis
70	F10	S100A8	S100 calcium binding protein A8	60B8AG, CAGA, CFAG, CGLA, CP-10, L1Ag, MA387, MIF, MRP8, NIF, P8	Phospholipidosis
71	F11	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	FADS5, MSTP008, SCD1, SCDOS	Steatosis
72	F12	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	AACT, ACT, GIG25, MGC88254	Phospholipidosis
73	G1	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	PAI, PAI-1, PAI1, PLANH1	Necrosis
74	G2	SKIL	SKI-like oncogene	SNO, SnoA, SnoI, SnoN	Necrosis
75	G3	SLC17A3	Solute carrier family 17 (sodium phosphate), member 3	NPT4	Hepatotoxicity
76	G4	SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	FLJ90380, GLUT3	Phospholipidosis
77	G5	SLC39A6	Solute carrier family 39 (zinc transporter), member 6	LIV-1	Necrosis
78	G6	SREBF1	Sterol regulatory element binding transcription factor 1	SREBP-1c, SREBP1, bHLHd1	Steatosis
79	G7	TAGLN	Transgelin	DKFZp686B01212, DKFZp686P11128, SM22, SMCC, TAGLN1, WS3-10	Phospholipidosis

80	G8	THRSP	Thyroid hormone responsive	LPGP1, Lpgp, MGC21659, S14, SPOT14	Hepatotoxicity
81	G9	TMEM2	Transmembrane protein 2	-	Necrosis
82	G10	TXNRD1	Thioredoxin reductase 1	GRIM-12, MGC9145, TR, TR1, TRXR1, TXNR	Hepatotoxicity, Nongenotoxic Hepatocarcinogenicity
83	G11	WIPI1	WD repeat domain, phosphoinositide interacting 1	ATG18, ATG18A, FLJ10055, WIPI49	Phospholipidosis
84	G12	YRDC	YrdC domain containing (E. coli)	DRIP3, FLJ23476, FLJ26165, IRIP, RP11-109P14.4, SUA5	Hepatotoxicity

Table adapted from: **http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-093Z.html (Sabiosciences 2017)**

Table 51: A summary of gene activity associated with different liver disease pathways

Pathology	Upregulated genes	Regulated genes	Downregulated genes
Cholestasis		ABCB1, ABCB4, ABCC2, ABCC3, ATP8B1, ICAM1, SLC51A, PDYN, RDX	
Steatosis	CD36, FASN, LPL, SCD		PPARA, SREBF1
Phospholipidosis	ASAH1, FABP1, HPN, LSS, MRPS18B, S100A8, SERPINA3, WIPI1	ABCB1, TIMM10B	SLC2A3, TAGLN
Hepatotoxicity	ALDOA, APEX1, BTG2, CASP3, CCNG1, CRYL1, DDIT4L, DNAJB11, DNAJC3, GADD45A, GCLC, GSR, HMOX1, HYOU1, KRT18, KRT8, NQO1, PLA2G12A, SLC17A3, TXNRD1, YRDC	ABCB11, TIMM10B, MAOB, PYGL	AVPR1A, BHMT, CA3, CXCL12, CYP1A2, FADS1, FMO1, HAO2, IGFALS, MBL2, RB1, THRSP
Nongenotoxic Hepatocarcinogenicity	ALDOA, APEX1, BTG2, CCNG1, CDKN1A, DDX39A, KRT8, KRT18, MRPS18B, TXNRD1		
Necrosis	CD68, COL4A1, IL6ST, IPO4, MAP3K6, NUS1, OSMR, PSME3, SERPINE1, SKIL, SLC39A6, TMEM2	CDKN1A, DDX39A	CDC14B, EMC9, FAM214A, L2HGDH, LGR5, MCM10, MLXIPL, RHBG

Table adapted from: http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-093Z.html (Sabioscience 2017)

Table 51 shows how the included genes are grouped together by disease pathway and what expression change would be expected in the disease pathway that they are associated with.

5.2.9 Lactic Dehydrogenase (LDH) Assay

For an initial assessment of toxicity levels, the amount of LDH release was assayed after 24 hours incubation with the extracts and paracetamol control. The In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma-Aldrich, St. Louis, Missouri, United States) was used for this purpose. The plate was centrifuged at 250 × *g* for 4 minutes to pellet the cells. A volume of medium from each test well was transferred to a 96 well plate. Equal volumes of LDH Assay substrate solution, LDH Assay dye and 1 × LDH Assay Cofactor Preparation were combined to obtain a LDH Assay Mixture. The next step was to add double the volume of LDH Assay Mixture to each volume of medium in the 96 well plate. The plate was covered in foil and incubated at room temperature for 20-30 minutes. The reactions were terminated at the end of the incubation period with 1/10 volume of 1 M HCl. The samples were then measured spectrophotometrically at 490nm. They were also measured at 690nm to measure the background. This background reading was then subtracted from the original reading at 490nm.

5.2.10 MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

A 12 mM MTT stock was made up by adding 5 mg of MTT (Invitrogen, Carlsbad, CA, USA) to 1 mL of phosphate buffered saline (ThermoFisher Scientific, Waltham, Massachusetts, United States) and mixed until dissolved. HepG2 cells at a low passage number (passage 6) were seeded into a 96 well plate at a density of 1 x 10^3 in medium free of phenol red. The cells were incubated at 37°C in a CO_2 incubator for 48 hours with a media change after 24 hours. The cells were then treated with the following extracts, in triplicate at three concentrations of 100 µg/ml, 20 µg/ml and 5 µg/ml, *Actaea racemosa, Actaea cimicifuga and Actaea dahurica.* Cells without treatment were used as a 100% viability control. After 24 hours of treatment the media with the extracts was removed and replaced with fresh media. In each well 10 µL of MTT reagent was applied and incubated for 6 hours at 37°C. The next step was to remove the MTT reagent and pipette 10 µL of acidified isopropanol to each well until the purple precipitate was dissolved. The plate was then read at 490 nm. The reading of the non

treated cells was counted as 100% viability and the viability of the treated cells was calculated from this.

5.3 Results and discussion

5.3.1 Preparation and analysis of ethanolic extracts

Firstly plant material (root and rhizome) for each of the included species (Sheng Ma *"A. dahurica",* Sheng Ma *"A. cimicifuga" and A. racemosa*) were sourced. It was not possible to source *A. heracleifolia* in large enough quantity which is why it was not included. The plant materials were respectively used to prepare ethanolic extracts. These extracts were prepared in the same way as commercially available products to best replicate what may be ingested.

Table 52: Information for the three manufactured 60% ethanol *Actaea* extracts

Labelled species	Sample number	Amount of raw material used (g)	Resulting extract amount (g)	Yield (%)	Extract number
A. racemosa	DR10-014-A	100.9	40.33	40.69	SW02-001-11
Sheng Ma "A. dahurica"	Ch.B.01230952	100	23.77	23.77	SW02-070-A
Sheng Ma "A. cimicifuga"	Ch.B.13884K116-A	100	21.38	21.38	SW02-070-B

These extracts and the raw plant material were assessed using DNA methods, HPTLC and using morphology.

5.3.1.1 DNA Analysis of the plant material used for the three *Actaea* 60% ethanol extracts

In order to be assured of the species identity of the raw materials, genetic sequencing was desirable and so DNA extraction was attempted:

Table 53: DNA extraction details for the three Actaea raw material samples – concentration obtained spectrophotometrically.

Labelled species name	Sample number	DNA extraction number	Concentration (ng/µL)
A. racemosa	DR10-014-A	BC050	62.3
A. dahurica	Ch.B.01230952	BC049	31.9
A. cimicifuga	Ch.B.13884K116-A	BC046	35.8

The DNA extracts were amplified using ITS1 and ITS4 primers. The resulting PCR products were sent for sequencing. The sequence data was checked using a BLAST search (NCBI, Maryland, USA). BC050 (*A. racemosa)* was a 99% match to *A. racemosa*. BC046 (Sheng Ma *"A. cimicifuga")* gave a poor quality sequence trace and was only an 89% match to *A. cimicifuga*. The sequence traces can be found in the appendix. BC049 (Sheng Ma "*A. dahurica"*) yielded a very poor sequence trace and was therefore not usable.

Each of the extracts was checked using the qPCR assay in Chapter 4: Development of a qPCR assay for the authentication of *A. racemosa* and detection of potential adulterants. The results are in Table 54.

Table 54: Results of qPCR testing with species specific and generic primers – C_t values shown

Implied species	DMU Extract Number	*A. racemosa* test AVG C_t		*A. dahurica* test AVG C_t		*A. cimicifuga* test AVG C_t	
		Generic Primer	*A. racemosa* primer	Generic Primer	*A. dahurica* Primer	Generic Primer	*A. cimicifuga* Primer
A. racemosa	**BC050**	**13.39**	**11.74**	**9.14**	**27.18**	**13.39**	**23.13**
A. dahurica	**BC049**	**18.91**	**28.01**	**13.86**	**13.84**	**18.91**	**20.69**
A. cimicifuga	**BC046**	**17.38**	**32.23**	**12.61**	**12.25**	**17.38**	**14.99**
Positive Controls							
A. racemosa*	**Arac5.9**	**28.69**	**28.78**	**N/A**	**N/A**	**N/A**	**N/A**
A. dahurica*	**Adah5.9**	**N/A**	**N/A**	**23.31**	**25.37**	**N/A**	**N/A**
A. cimicifuga*	**Acim5.9**	**N/A**	**N/A**	**N/A**	**N/A**	**25.61**	**23.81**

C_t values less than 35 were deemed to be significant amplification.

Table 55 shows the calculated $\Delta\Delta C_t$ values and summary of test results.

Table 55: Results of qPCR testing of the Actaea raw material samples showing $\Delta\Delta C_t$ figures – identifications shown

Implied species	DMU Extract Number	ITS Barcode Sequence	*A. racemosa* qPCR $\Delta\Delta C_t$	*A. dahurica* qPCR $\Delta\Delta C_t$	*A. cimicifuga* qPCR $\Delta\Delta C_t$	Conclusions
Actaea racemosa	**BC050**	***A. racemosa***	**-1.7**	**16.0**	**11.6**	*A. racemosa*
Actaea dahurica	**BC049**	**Not able to sequence**	**9.0**	**-2.1**	**3.6**	*A. dahurica* and possibly *A. cimicifuga* present
Actaea cimicifuga	**BC046**	**Poor quality sequence data**	**14.8**	**-2.4**	**-0.6**	*A. cimicifuga* and possibly *A. dahurica* present

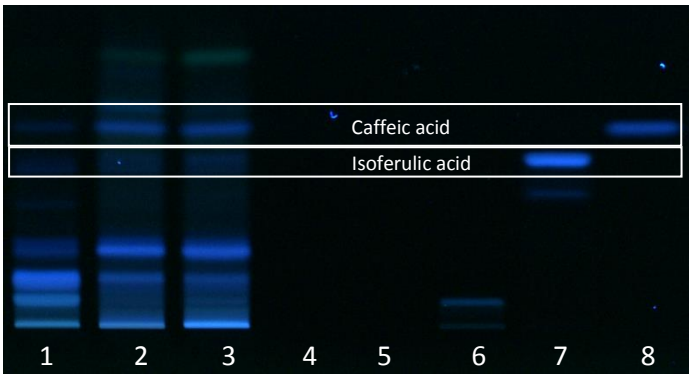
$\Delta\Delta C_T$: $[(C_t \text{ specific} - C_t \text{ generic})_{sample} - (C_t \text{ specific} - C_t \text{ generic})_{reference}]$. A value of 0.0 +/-1.0 is a clear positive result. A value > 6.0 is a clear negative. **Barcode sequence**. ITS - The nuclear ribosomal internal transcribed spacer barcode sequence. The nearest hit by BLAST search is indicated.

So it would appear from these results that *A. racemosa* is the correct identity for the BC050 extraction as the sequence matches well with what is available on GenBank. The *A. cimicifuga* labelled material had a positive result for *A. cimicifuga* but *A. dahurica* was also detected in the qPCR assay. The ITS sequence data was checked using BLAST and the top hit was *A. dahurica* but only at 89% which is not a convincing match. Further down the match list *A. cimicifuga* is also shown at 87%. The sequence data was not of great quality and so this is inconclusive. It does appear that the material could be a mixture of both *A. dahurica* and *A. cimicifuga.* The *A. dahurica* labelled material also appears to contain both *A. dahurica* and *A. cimicifuga* but this cannot be backed up with sequencing as it was not possible to obtain a good product with ITS primers. It is not possible to reliably sequence mixed sample which could explain why it was not possible in this case; i.e. the material is mixed. The supplier of these materials is based in China and the materials are destined to be used in the Traditional Chinese medicine, Sheng Ma. The Chinese pharmacopoeia has a test that does not discriminate between *A. dahurica* or *A. cimicifuga* as both species are acceptable for use in Sheng Ma. This marker is Isoferulic acid.

5.3.1.2 High Performance Thin Layer Chromatography of the three *Actaea* extracts

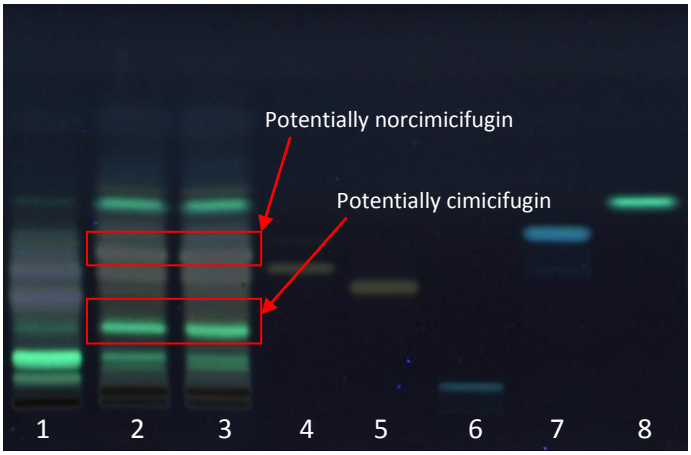
The CAMAG developed method for *Actaea racemosa* identification and adulterant detection was used for this purpose. There are three techniques; plant acids, triterpene glycosides and a method combing the two.

A.



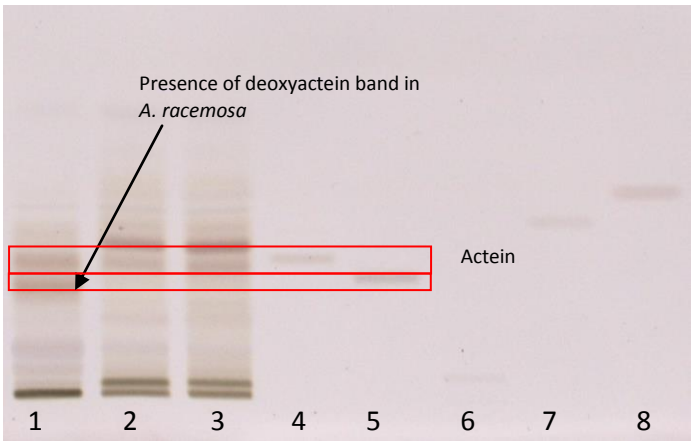
1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

B.



1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

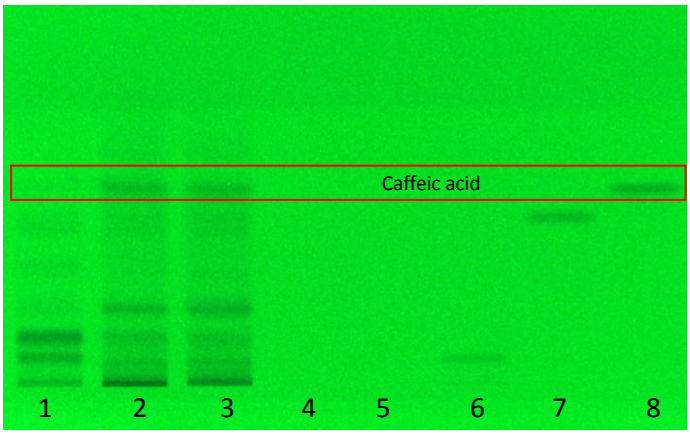
C.



1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

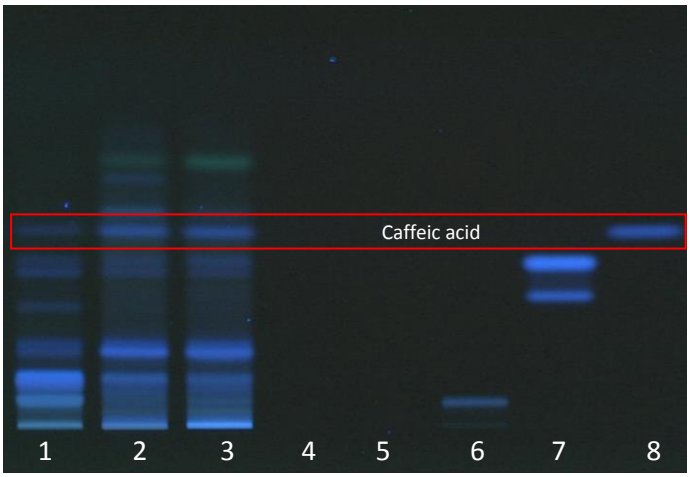
Figure 58: HPTLC plates using the Black Cohosh combined detection method developed by CAMAG Laboratories. A. 366 nm prior to derivitisation, B. 366 nm after derivitisation and C. white light after derivitisation. Key bands annotated.

A.



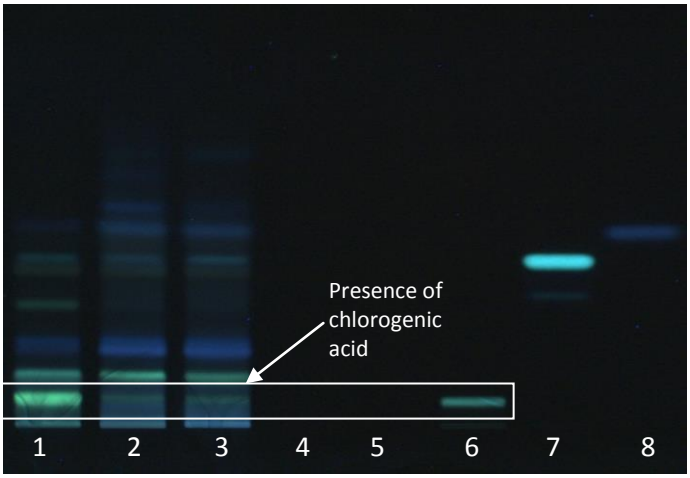
1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

B.



1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

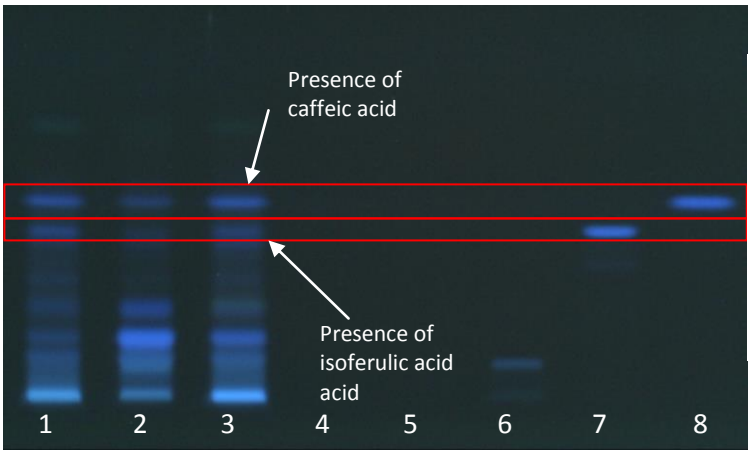
C.



1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

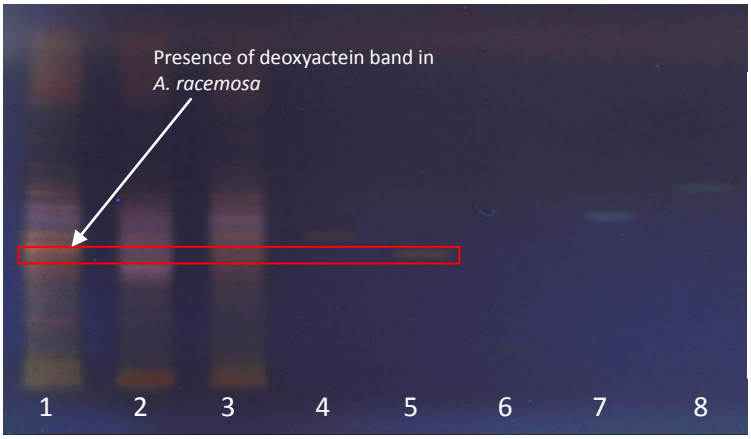
Figure 59: HPTLC plates using a plant acid method developed by CAMAG laboratories, A. 254 nm before derivitisation, B. 366 nm before derivitisation and C. 366 nm after derivitisation. Key bands annotated.

A.



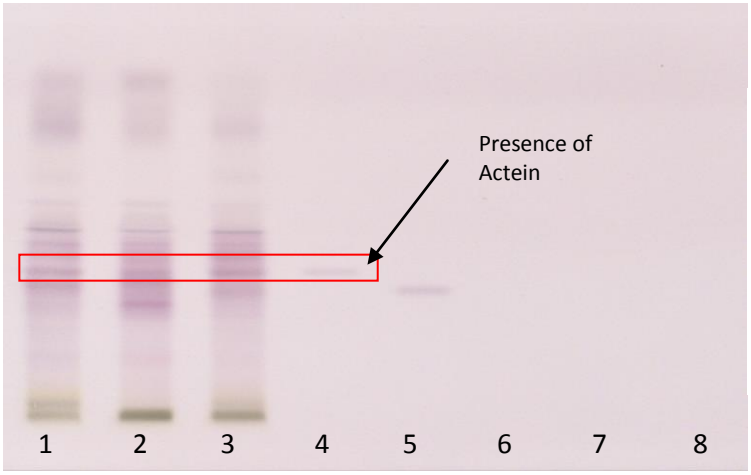
1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

B.



1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

C.



1 – *A. racemosa* extract
2 – Sheng Ma *A. dahurica* extract
3 – Sheng Ma *A. cimicifuga* extract
4 – Actein
5 – Deoxyactein
6 – Chlorogenic acid
7 – Isoferulic acid
8 – Caffeic acid

Figure 60: HPTLC plates using a triterpene method developed by CAMAG laboratories, A. 366nm before derivitisation, B. 366nm after derivitisation and C. white light following derivitisation. Key bands annotated.

The main difference between *Actaea racemosa* and the other species is the deoxyactein band is present. This is displayed in Figure 60 Plate B.

According to the sample plate in the method developed by CAMAG (Shown in Figure 52), for *Actaea cimicifuga*, chlorogenic acid, caffeic acid and actein are present. These bands can be seen across the

different methods in both Sheng Ma extracts (Isoferulic acid and caffeic acid shown in Figure 60 plate A, chlorogenic acid shown in Figure 59 plate C) , although actein seems to have a stronger presence in the material labelled as *Actaea cimicifuga* (Shown in Figure 60 plate C). For *Actaea dahurica* there were no reference markers included on the plate that are present in *Actaea dahurica* and not in *Actaea cimicifuga* although such reference markers do exist as shown by He at al., 2006. There are no specific unique markers to *A. dahurica,* rather the pattern of markers are diagnostic for identification. Cimicifugic acid A, cimicifugic acid B, Fukinolic acid, 2-Feruloyl piscidic acid and 2-Isoferuloyl piscidic acid are such examples of compounds present in *A. dahurica* and not *A. cimicifuga.* These ideally would have been utilised but they were unavailable at the time of conducting the technique. The recommended markers for the combined CAMAG method include cimicifugin and norcimicifugin. These were not available at the time of conducting the experiment but the predicted bands have still been annotated on Figure 58 plate B which appear to be present in both Sheng Ma extracts as would be expected in either *A. dahurica* or *A. cimicifuga*. Both of the materials labelled respectively as *Actaea dahurica* and *Actaea cimicifuga* give an almost identical profile on the plate. The markers that match to the reference markers are not uniquely diagnostic for *A. cimicifuga* or *A. dahurica* as a whole but they can be used as a guide. The profiles are also different from *A. racemosa.* It would appear that both of the Sheng Ma extracts could be a mix of both *A. dahurica* and *A. cimicifuga*, which supports the results of the DNA based methods in 5.3.1.1 DNA Analysis of the plant material used for the three *Actaea* 60% ethanol extracts.

5.3.1.3 Morphological examination of *Actaea* root material

All three plant materials were analysed by the Pharmacognosy department at Schwabe Pharma GmbH. The botanical identification of the *Actaea racemosa* material was straightforward flowering plants were available as the source for this material. The other two materials, Sheng Ma "*Actaea dahurica"* and Sheng Ma "*Actaea cimicifuga"* were very difficult for the department to clearly identify. There were features present in the *Actaea cimicifuga* material that were characteristic of the species. There were also important features missing and as such a clear identification could not

be given. The *Actaea dahurica* material could also not be clearly identified due to the processing it had been through. For the two species; *Actaea dahurica* and *Actaea cimicifuga* fertile flowering above ground plants would be required for identification. This further supports the need for more robust tests to be developed for these materials, where identification can be given.

Table 56: A table to summarise all of the methods utilised to attempt identification of the raw plant materials used to manufacture 60% ethanol extracts

Labelled species	HPTLC	DNA tests	Morphology	Comments
Actaea racemosa	*Actaea racemosa*	*Actaea racemosa*	*Actaea racemosa*	All test identify as *Actaea racemosa*
Actaea dahurica	Could be *Actaea dahurica* or *Actaea cimicifuga*	Could be *Actaea dahurica* or *Actaea cimicifuga*	Not able to identify	Could be *Actaea dahurica* and *Actaea cimicifuga*
Actaea cimicifuga	Likely *Actaea cimicifuga* could also contain *Actaea dahurica*	Could be *Actaea dahurica* and *Actaea cimicifuga*	Some characteristics of *Actaea cimicifuga* present	Could be *Actaea dahurica* and *Actaea cimicifuga*

5.3.2 Treatment and gene expression analysis of HepaRG™ Cells

The next stage was to culture and treat HepaRG™ cells and investigate the effect of the extracts after 24 hours of treatment via gene expression analysis.

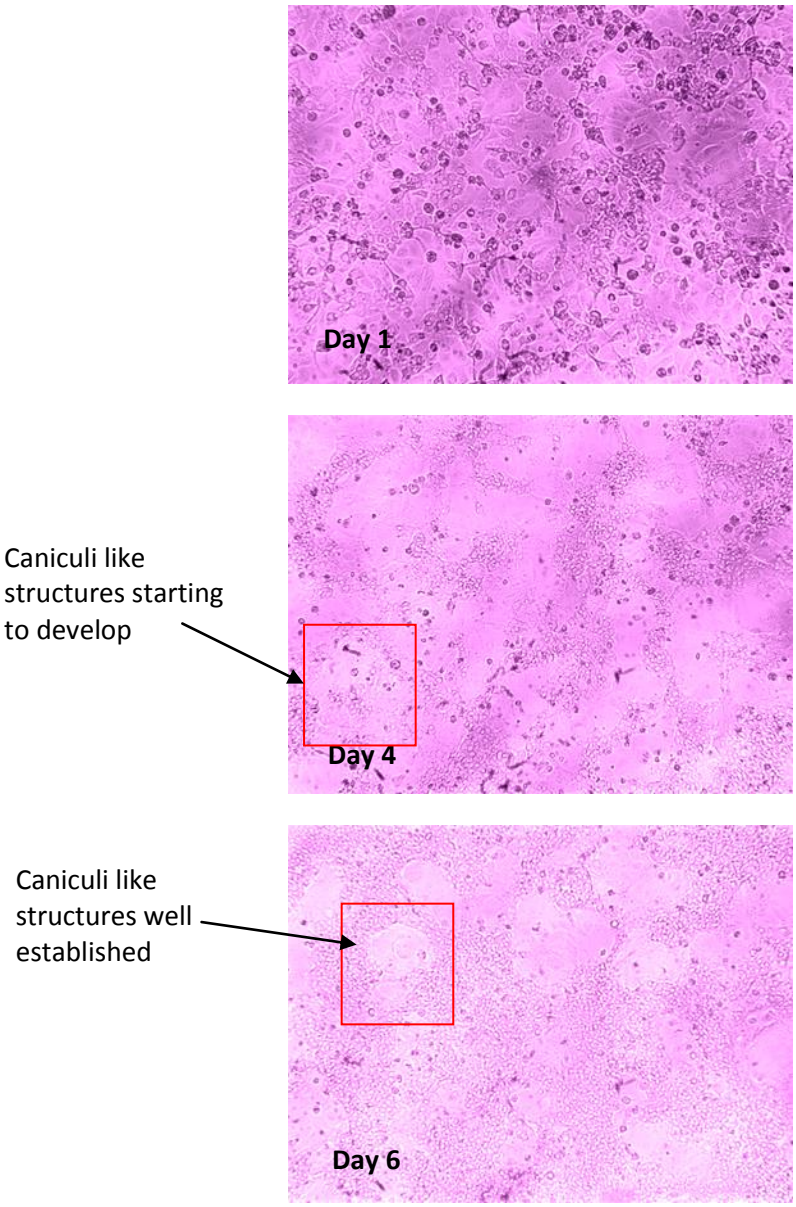


Figure 61: Photomicrographs of HepaRG™ cells in culture – Day 1, 4 and 6 displayed showing key features

The cells performed as was hoped during the 7 days of culturing. On day 1 the cells had started to settle into a monolayer, this is not clear in the photomicrograph but the cells had adhered and settled into the collagen coating of the 24 well plate. By day 4 bile caniculi were starting to become apparent and by day 6 the cells were well established with well-formed caniculi like structures. There

were no signs of infection or contamination. The cells were cultured up to day 7 and then treatment could begin.

5.3.2.1 Pilot experiment of treating the HepaRG™ cells with three different *Actaea* extracts

The cells were cultured in a 24 well plate and treated with the extracts. After 24 hours, RNA was extracted from each of the wells; duplicate samples were pooled together and converted to cDNA. The cDNA was analysed with the human hepatotoxicity RT2 Profiler array.

After the first array was completed the threshold was manually adjusted as outlined in 5.2.8 Preparation and analysis of Quantitative Polymerase Chain Reaction arrays. All subsequent arrays were adjusted to the same threshold.

Once all the arrays were completed, the C_t data was imported to an Excel spreadsheet and analysed using the Qiagen RT^2 profiler web based software.

The first step on the analysis software was to check through the quality control checks.

There are three things which are checked; PCR array reproducibility, reverse transcription efficiency and genomic DNA contamination. All samples passed in PCR array reproducibility and genomic DNA contamination but there was an enquiry for reverse transcription efficiency. This number must be less than 5 and is calculated from the average of RTC controls minus the average of the PPC controls. This is summarised in Table 57.

Table 57: A table to show the summary of QC tests that required attention.

Test Performed	Control Group	Acetaminophen	*A. racemosa*
ΔC_t (AVG RTC - AVG PPC)	5.03	5.09	5.49
Result	Inquiry	Inquiry	Inquiry

The next stage in the process was to perform normalisation using the housekeeping genes. The rule for this step was to include genes where differences in the arithmetic mean of the C_t values were not more than 1 between the different arrays. The C_t values of the housekeeping genes are shown in

Table 58. The housekeeping genes assessed as part of this experiment were; ACTB – Actin Beta, B2M – Beta 2 Microglobulin, GADPH - Glyceraldehyde-3-Phosphate Dehydrogenase, HPRT1 - Hypoxanthine Phosphoribosyltransferase 1 and RPLP0 – Large Ribosomal Protein. These genes are selected as they encode proteins that have critical roles in basic cell maintenance. These genes maintain constant expression in all conditions and thus show a minimum variability between samples. The C_t values that the housekeeping genes give following qPCR cycling are subtracted from the C_t values from each of the 84 test genes. This is termed as normalisation. The genes are used to normalise the data only when there is minimal deviation of the values between samples. The average of the genes HPRT1 and RPLPO were used for normalisation within this experimental set.

Table 58: C_T values obtained from qPCR cycling of cDNA samples from the cultured cells – housekeeping gene controls

Position	Gene Symbol	Control (Non-treated)	APAP	*A. racemosa*	*A. dahurica*	*A. cimicifuga*	Mean
H01	ACTB	16.95	18.40	17.02	16.60	16.94	17.182
H02	B2M	19.37	20.37	19.31	19.65	19.59	19.658
H03	GAPDH	18.58	20.98	18.43	18.12	18.13	18.848
H04	HPRT1	25.78	25.97	25.37	24.80	25.31	25.446
H05	RPLP0	18.69	18.93	18.25	18.08	18.25	18.44

Table 59: A table depicting fold regulation – a comparison of the control group to the test groups – figures exceeding 1 show increased expression and negative figures show decreased expression of the genes tested.

Position	Gene Symbol	APAP	*A. racemosa*	*A. dahurica*	*A. cimicifuga*
A01	ABCB1	1.181	-1.3566	-1.5263	-1.3566
A02	ABCB11	-8.6939	-4.5631	-3.8106	-4.0558
A03	ABCB4	-6.5432	-3.3404	-2.8679	-3.0314
A04	ABCC2	-9.9177	-2.4623	-1.5476	-1.7654
A05	ABCC3	-3.0105	-1.6021	-1.9862	-1.8921
A06	ALDOA	-2.0279	-1.4439	-1.2058	-1.2311
A07	APEX1	-1.9725	-1.6245	-1.5052	-1.6702
A08	ASAH1	1.057	-1.9185	-1.7291	-1.9053
A09	ATP8B1	1.9319	-3.3404	-1.9185	-2.5847
A10	AVPR1A	-1.7654	-1.2483	-1.0792	-1.8661
A11	BHMT	-1.454	-2.0705	-1.6021	-1.1019
A12	BTG2	-3.0105	-1.057	1.181	-1.0497
B01	CA3	-1.1975	-3.0951	-2.2501	-2.4116
B02	CASP3	-1.1019	-1.8404	-1.366	-1.366
B03	CCNG1	-2.0139	-1.5052	-1.5692	-1.6935

B04	CD36	-2.042	-6.9644	-2.2191	-1.3851
B05	CD68	-2.7511	-1.0425	1.3472	1.057
B06	CDC14B	-2.114	-2.514	-2.1585	-2.2501
B07	CDKN1A	1.1567	-1.4142	-1.0943	-1.1251
B08	COL4A1	2.0705	-2.1287	-1.5052	-1.6133
B09	CRYL1	-1.1892	-3.0525	-2.4453	-2.6208
B10	CXCL12	1.181	-1.3566	-1.5263	-1.3566
B11	CYP1A2	-1.0281	-9.2535	-1.6245	3.7581
B12	DDIT4L	-2.1886	-2.6759	-3.9449	-3.5064
C01	DDX39A	-2.0705	-1.257	1.2058	-1.1173
C02	DNAJB11	1.1019	-1.3947	-1.5583	-1.5476
C03	DNAJC3	-1.1487	-1.3755	-1.8025	-1.6472
C04	FABP1	-25.2813	-9.9177	-36.0019	-13.4543
C05	FADS1	-1.0281	-2.0562	-1.6245	-1.5692
C06	EMC9	1.3472	-1.4743	-1.6133	-1.5801
C07	FASN	-1.1567	-3.0105	-2.969	-2.5491
C08	FMO1	-6.0629	-4.0000	-16.3362	-2.8679
C09	TIMM10B	1.1975	-1.257	-1.4044	-1.1892
C10	GADD45A	5.5022	-1.3013	-1.1019	-1.181
C11	GCLC	1.3947	-1.879	-2.2038	-2.1435
C12	GSR	-1.1647	-1.879	-1.7654	-1.4044
D01	HAO2	-106.1529	-12.7286	-26.9087	-9.7811
D02	HMOX1	2.2658	-1.7777	-1.5476	-1.6358
D03	HPN	-10.2674	-1.8277	-3.8371	-2.9079
D04	HYOU1	2.3295	-1.1567	-1.454	-1.3195
D05	ICAM1	-2.4284	2.2815	1.5911	1.4641
D06	IGFALS	-2.5315	-1.5476	-4.0000	-1.2058
D07	IL6ST	-1.0000	-1.3851	-1.4641	-1.5369
D08	IPO4	1.2397	-1.1647	1.0497	-1.0281
D09	FAM214A	-2.0139	-1.4743	-1.9053	-1.5911
D10	KRT18	-2.3457	-1.0644	-1.0425	-1.2058
D11	KRT8	-2.114	-1.6586	-1.0943	-1.4948
D12	L2HGDH	-1.3566	-2.2191	-1.5476	-1.5583
E01	LGR5	-5.3147	-4.1125	-9.5798	-8.515
E02	LPL	-1.014	-1.021	-1.2058	-1.1487
E03	LSS	-1.9862	-2.0705	-1.7532	-1.8532
E04	MAOB	-4.5631	-3.2944	-2.8481	-2.5847
E05	MAP3K6	-2.7702	-1.3195	1.0000	-1.2397
E06	MBL2	-10.7034	-3.2266	-3.3636	-1.8661
E07	MCM10	-3.4822	-1.3287	2.6208	1.6245
E08	MLXIPL	-1.2142	-2.8481	-3.5308	-2.4967
E09	MRPS18B	-1.2924	-1.1728	-1.2058	-1.2058
E10	NQO1	-4.1125	-1.5476	-1.181	-1.6358
E11	NUS1	-1.3947	-1.257	-1.3195	-1.3287
E12	OSMR	-1.8025	1.2483	-1.0792	-1.0497
F01	SLC51A	-25.1067	-1.0425	-1.0000	1.0353
F02	PDYN	1.181	-1.3566	4.724	3.8906
F03	PLA2G12A	1.1408	-1.7171	-1.5052	-1.6702
F04	PPARA	-1.1728	-1.9319	-1.8404	-2.114
F05	PSME3	-1.0497	-1.5911	-1.2397	-1.4044
F06	PYGL	-4.2575	-1.7053	-1.257	-1.434
F07	RB1	-1.6818	-1.6133	-1.2924	-1.6021
F08	RDX	-1.3195	-1.815	-1.2834	-1.5157
F09	RHBG	-2.2346	-3.6553	-2.114	-1.6245
F10	S100A8	-9.3827	1.0867	-3.9177	-1.4743
F11	SCD	-2.0279	-1.9319	-1.6702	-1.6358
F12	SERPINA3	-5.5022	1.6472	-1.1647	1.0644
G01	SERPINE1	-1.2142	1.0353	1.5263	1.1019
G02	SKIL	-1.7777	-2.0994	-1.8404	-2.5491
G03	SLC17A3	-6.2767	-2.969	-2.2501	-1.6133
G04	SLC2A3	1.181	1.0792	-1.5263	1.0497
G05	SLC39A6	1.4241	-1.4439	1.0353	-1.2142
G06	SREBF1	-4.1125	-2.1435	-2.114	-2.0279
G07	TAGLN	-1.7171	-1.5052	-1.5692	-1.8921

G08	THRSP	-3.1602	-16.7955	-28.84	-15.6707
G09	TMEM2	-1.0943	-1.1173	-1.3013	-1.2142
G10	TXNRD1	1.7777	-2.4116	-1.7532	-1.7171
G11	WIPI1	1.6702	-1.0425	-1.7053	-1.6133
G12	YRDC	1.6245	-1.4641	-1.5052	-1.454

As the first experiment was a pilot to check the feasibility of the set of experiments there were not biological replicates; the RNA was pooled from the duplicates. The final experiment will be made up of biological triplicates which will each be analysed by a separate array.

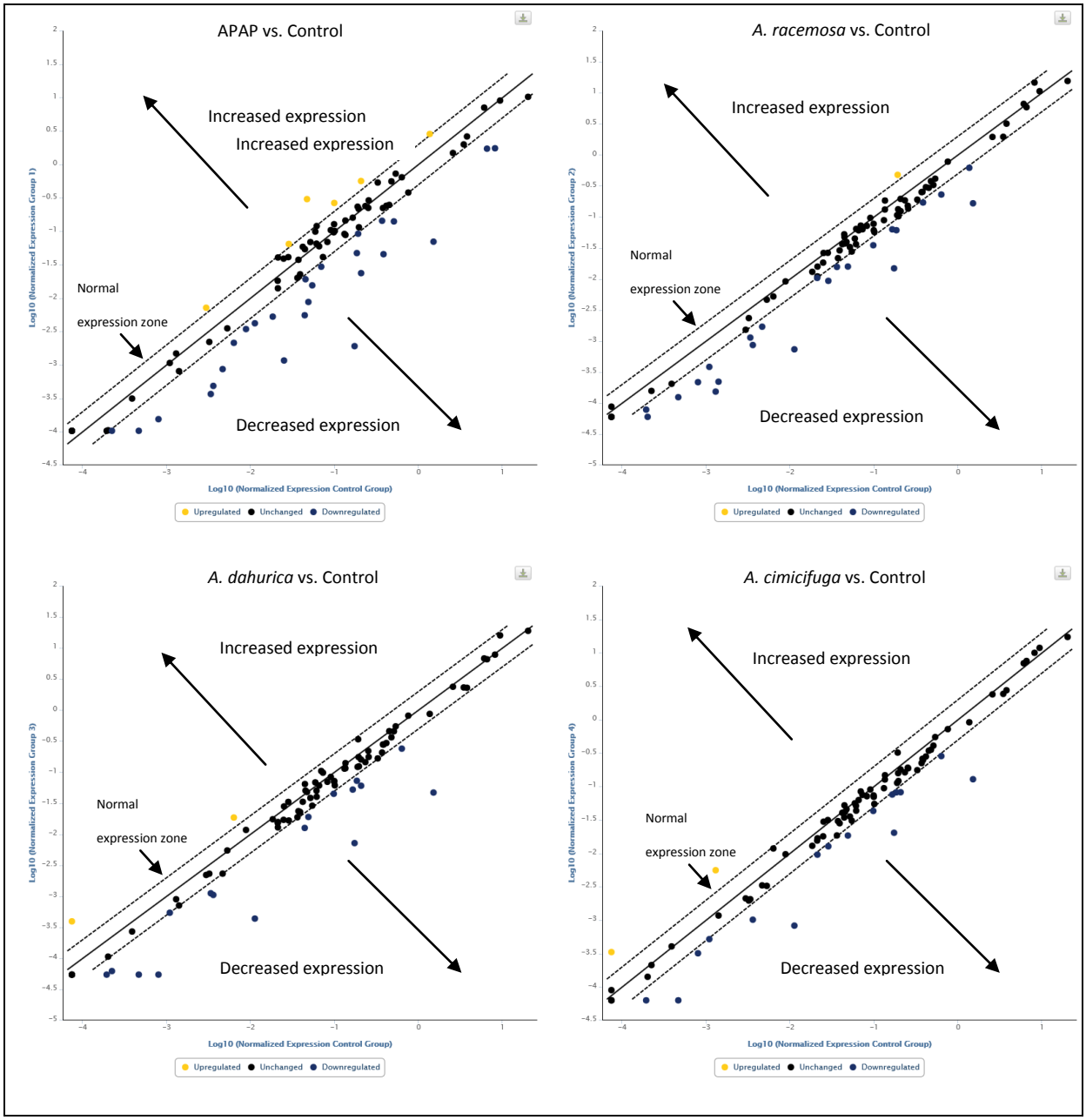


Figure 62: Scatter plots giving an overall view of the effects of the extracts on regulation of the assessed genes – increased expression of genes are shown as yellow dots, decreased expression of genes is shown as blue dots and normal expression of genes is shown as black dots within the dotted lines. All expression changes are as compared to the control.

The scatter plots in Figure 62 give a quick visual on the number and changes in gene expression that have occurred in the experiments. As can be seen in the scatter plot there was a lot of change in expression of the genes analysed for the APAP treated cells, the majority of the changes were decreases in expression. The scatter plot displays the over expressed genes with yellow dots, the under expressed genes with blue dots and the genes that are not significantly over or under expressed are black dots. The fold regulation values are used for creating these plots.

Sheng Ma *A. cimicifuga* and Sheng Ma *A. dahurica* have a very similar profile. Considering that they also had the same chemical profile on the HPTLC plate this is not surprising (Section 5.3.1.2 High Performance Thin Layer Chromatography of the three *Actaea* extracts).

Following on from the successful completion of the pilot study a full scale experiment was designed. Having proven that the method was capable of measuring the hepatotoxicity of the included extracts further investigation of the Black Cohosh and Sheng Ma extracts was embarked upon.

5.3.2.2 Biological triplicates of two *Actaea* species at three concentrations

On the 7th day of culturing, the cells were treated with the following extracts and preparations;

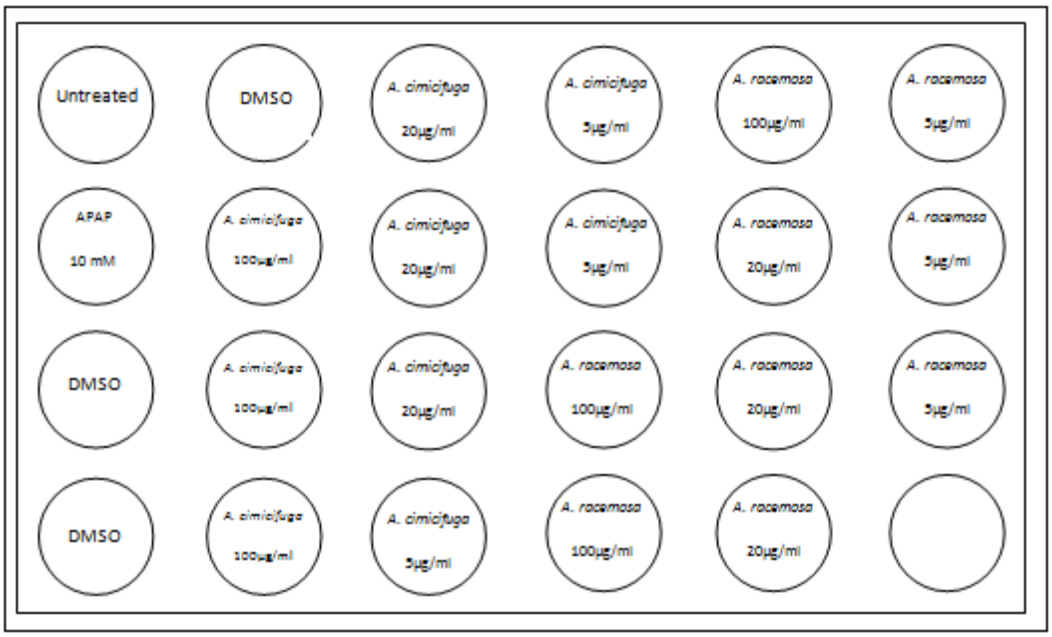


Figure 63: The layout of the 24 well plate used for culturing and treating the HepaRG™ for the final experiment

Following 24 hours of treatment RNA was extracted from each well respectively. The quality and concentration of the RNA was assessed.

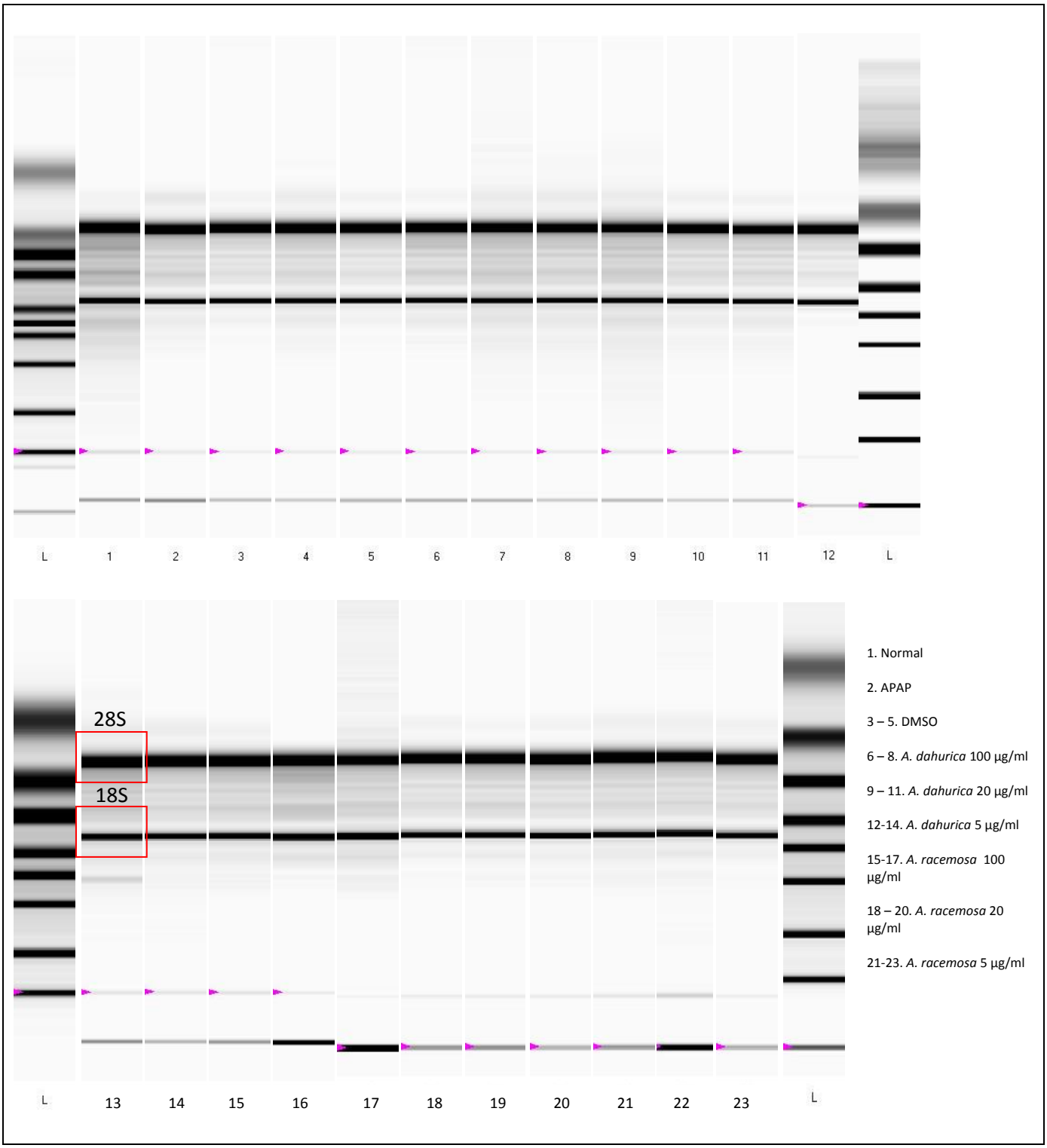


Figure 64: Results of extracted RNA analysis on a BioRad RNA Chip – the 28S and 18S subunits shown indicating good quality RNA extractions

The results show that the RNA is of good quality as the 18S and 28S regions are present. The 18S and 28S bands are intense indicating that there is a good quantity of RNA present and another point worth noting is the ratio of 28S to 18S appears to be 2:1 a well-known indication of good quality.

There are no bands above the 28S band indicating that there is no genomic DNA contamination as genomic DNA runs slower on the gel.

The next step was to quantify the RNA as this is useful for the cDNA synthesis step.

Table 60: Quantification data for the RNA extractions – obtained using spectrophotometry

Sample	Description	Concentration (ng/µL)	A260	A280	A260/A280
1	Normal control	155.39	3.884732	1.840235	2.110997
2	Acetaminophen 10mM	93.69	2.342211	1.12603	2.080061
3	DMSO	165.34	4.133544	1.96246	2.106308
4	DMSO	183.65	4.59135	2.213783	2.073984
5	DMSO	156.18	3.904527	1.88203	2.074636
6	*Actaea dahurica 100µg/ml*	171.62	4.290485	2.057635	2.085153
7	*Actaea dahurica 100µg/ml*	137.79	3.444759	1.652092	2.08509
8	*Actaea dahurica 100µg/ml*	185.90	4.647447	2.229838	2.084209
9	*Actaea dahurica 20µg/ml*	143.46	3.586533	1.704871	2.103698
10	*Actaea dahurica 20µg/ml*	199.23	4.980789	2.440569	2.040831
11	*Actaea dahurica 20µg/ml*	168.96	4.223925	2.012511	2.098834
12	*Actaea dahurica 5µg/ml*	147.39	3.684646	1.74898	2.106739
13	*Actaea dahurica 5µg/ml*	162.34	4.058522	1.952972	2.078126
14	*Actaea dahurica 5µg/ml*	135.02	3.375463	1.624415	2.077957
15	*Actaea racemosa 100µg/ml*	41.89	1.047333	0.523771	1.999602
16	*Actaea racemosa 100µg/ml*	164.72	4.117878	1.981429	2.078237
17	*Actaea racemosa 100µg/ml*	150.40	3.760097	1.781039	2.111182
18	*Actaea racemosa 20µg/ml*	160.56	4.013945	2.220012	1.808074
19	*Actaea racemosa 20µg/ml*	134.04	3.350977	1.620174	2.068282
20	*Actaea racemosa 20µg/ml*	188.34	4.708404	2.328769	2.021843
21	*Actaea racemosa 5µg/ml*	154.41	3.860323	1.874301	2.059607
22	*Actaea racemosa 5µg/ml*	136.18	3.404568	1.625911	2.093945
23	*Actaea racemosa 5µg/ml*	122.10	3.052606	1.478947	2.06404

The A260/A280 ratio is an indication of RNA purity. A figure of around 2 is accepted as 'pure' and the majority of the samples are close to this figure. Anything considerably lower could indicate contamination with proteins or phenols for example. The results look encouraging at this point.

The next step now is to convert the RNA to cDNA using the First Strand Kit. The kit uses an enzyme, reverse transcriptase, to convert the RNA into cDNA. There is a built in genomic DNA digestion step within the kit to clear the RNA extraction of any potential genomic DNA contamination prior to the

conversion to cDNA. Although the RNA chip results indicate no genomic DNA contamination is present it is still worthwhile to take every precaution possible as any contamination would be detrimental to the gene expression results. There is a genomic DNA control on the array also for absolute confidence.

Depending on the concentration of the RNA samples various volumes were used as a template in the cDNA synthesis reactions. The arrays could be completed now that the cDNA was ready.

Once the first array was complete, the threshold was manually altered as previously described in the pilot experiment.

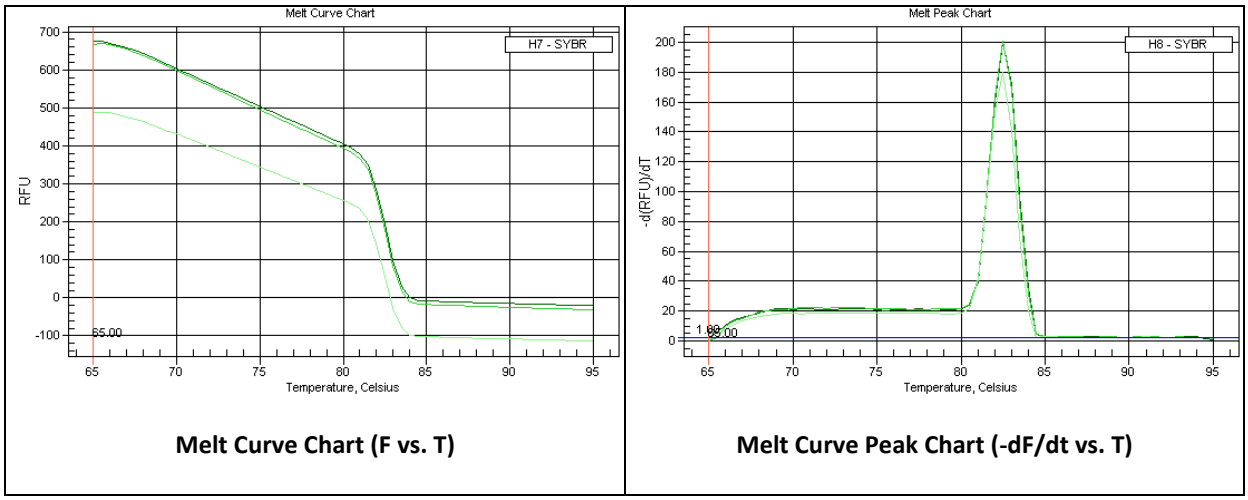


Figure 65: Melt curve plots for the triplicate reverse transcription controls. On the left is the dissociation curve of measured fluorescence plotted against temperature, on the right is the negative first derivative plot which displays the rate of change of fluorescence vs. temperature – the point of maximum rate of change shows the melting point of the amplicon

The melt curves of the reverse transcription controls were studies as shown in Figure 65 to ascertain that one peak is seen indicating one product is present and the control has been successfully amplified. Each of the triplicates has been included.

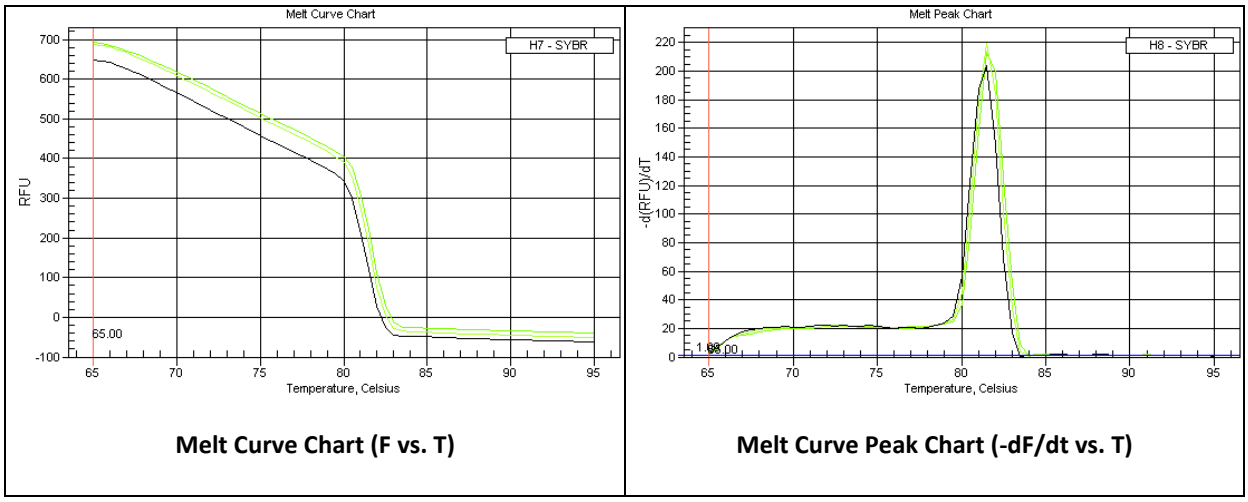


Figure 66: Melt curve plots for the positive PCR Control. On the left is the dissociation curve of measured fluorescence plotted against temperature, on the right is the negative first derivative plot which displays the rate of change of fluorescence vs. temperature – the point of maximum rate of change shows the melting point of the amplicon

Each triplicate has the same melting point displaying that the same product is being formed in each reaction. Each triplicate also displays one peak showing that a single product is being formed in the reaction.

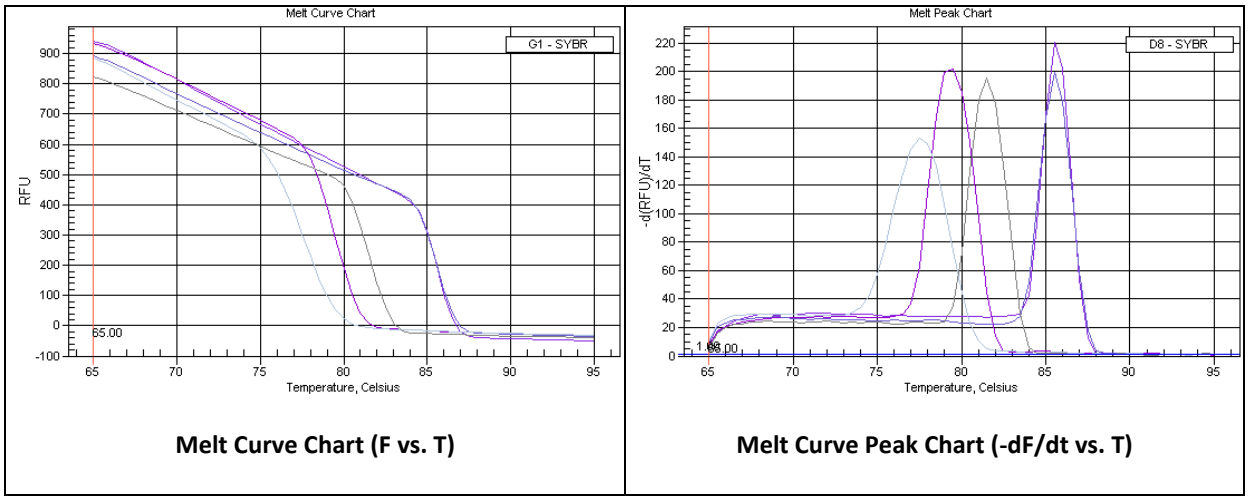


Figure 67: Melt curve analysis for the Housekeeping genes. On the left is the dissociation curve of measured fluorescence plotted against temperature, on the right is the negative first derivative plot which displays the rate of change of fluorescence vs. temperature – the point of maximum rate of change shows the melting point of the amplicon

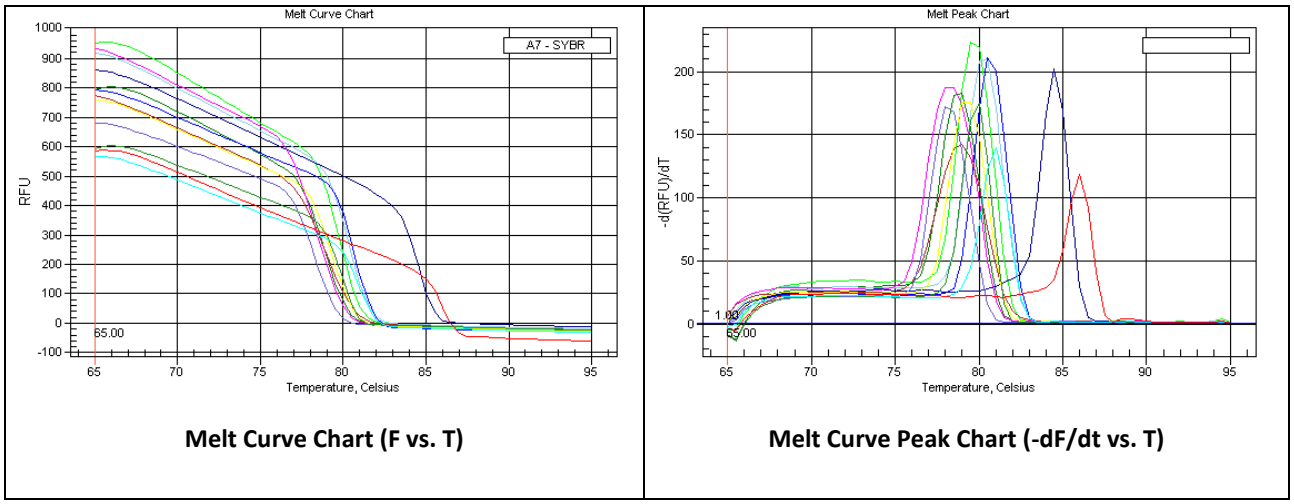


Figure 68: Melt curve analysis of 12 of the genes of interest. On the left is the dissociation curve of measured fluorescence plotted against temperature, on the right is the negative first derivative plot which displays the rate of change of fluorescence vs. temperature – the point of maximum rate of change shows the melting point of the amplicon

The melt curves are just a selection of what was carried out but are representative of the success of all the plates carried out. The desired outcome was to produce reactions with a single peak on the melt curve. This indicated that one product was produced.

Once all the arrays were completed, the next step was to import all the raw data from the qPCR cycler into the online analysis tool (Available from: http://www.qiagen.com/gb/shop/genes-and-pathways/data-analysis-center-overview-page/). Once all the data was imported it was necessary to choose housekeeping genes for normalisation and review all the QC data. The QC data comes from the controls built into the plate. There is a genomic DNA control which if successful will not produce a product. Fortunately, the C_t value for every plate was more than 35. There were three wells used for reproducibility testing and a further three wells used for a reverse transcription control. All of the QC tests were a pass for all samples.

Table 61: C_t values for housekeeping genes – these values are used for normalisation

Position	Gene Symbol	Control Gene	Control Group			Group 1			Group 2			Group 3			Group 4			Group 5			Group 6		
			3 DMSO	4 DMSO	5 DMSO	15* CR 100	16 CR 100	17 CR 100	18 CR 20	19 CR 20	20 CR 20	21 CR 5	22 CR 5	23 CR 5	6 CD 100	7 CD 100	8 CD 100	9 CD 20	10 CD 20	11 CD 20	12 CD 5	13 CD 5	14 CD 5
H01	ACTB	No	17.62	17.62	17.52	19.19	18.03	17.78	17.79	18.62	17.74	17.49	17.49	17.96	17.08	17.60	17.33	17.75	16.83	17.70	17.68	17.45	17.58
H02	B2M	Yes	18.87	18.69	19.21	21.30	19.34	19.20	19.03	20.07	19.17	19.01	19.14	19.35	18.80	19.40	18.67	19.17	18.43	19.11	19.12	18.94	19.17
H03	GAPDH	Yes	19.79	19.82	19.11	21.66	19.63	19.65	19.83	20.40	19.17	19.13	19.06	19.58	18.96	19.42	19.31	19.81	18.92	19.93	19.76	19.62	19.45
H04	HPRT1	Yes	25.45	25.28	25.26	26.78	25.80	25.92	25.88	26.67	25.75	25.61	25.68	25.91	25.36	25.80	25.40	25.72	25.17	25.60	25.68	25.74	25.75
H05	RPLP0	No	18.24	18.07	18.22	20.04	18.53	18.25	18.35	19.02	18.85	18.15	18.23	18.69	18.03	18.48	17.78	18.30	17.61	18.27	18.30	18.19	18.44
Arithmetic Mean			21.37	21.26	21.19	23.25	21.59	21.59	21.58	22.38	21.36	21.25	21.29	21.61	21.04	21.54	21.13	21.57	20.84	21.55	21.52	21.43	21.46
Avg. of Arithmetic Mean			21.27			22.14			21.77			21.38			21.24			21.32			21.47		

*Greyed out sample was not included in the analysis as the C_t values of the housekeeping genes deviated from the other samples too greatly.

Table 62: QC data for all arrays – including genomic DNA control, reverse transcription control and PCR array reproducibility control

Test Performed	3 DMSO	4 DMSO	5 DMSO	15 CR 100	16 CR 100	17 CR 100	18 CR 20	19 CR 20	20 CR 20	21 CR 5	22 CR 5	23 CR 5	6 CD 100	7 CD 100	8 CD 100	9 CD 20	10 CD 20	11 CD 20	12 CD 5	13 CD 5	14 CD 5
PCR Array Reproducibility																					
Average Ct (PPC)	19.52	19.57	19.77	19.57	20.27	19.62	19.89	19.91	19.85	19.50	19.82	19.8	19.91	19.93	20.12	20.54	19.70	19.85	19.93	21.38	20.02
Result	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Reverse Transcription Control (RTC)																					
Delta C_T (AVG RTC - AVG PPC)	3.5	3.4	3.5	3.9	3.2	3.4	3.4	3.2	3.2	3.4	3.4	3.5	3.4	3.2	3.4	3.3	3.5	3.4	3.9	1.7	2.6
Result	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Genomic DNA Contamination (GDC)																					
C_T(GDC)	0	37.94	37.61	35	35	35	35	35	35	35	35.91	35	35.81	35	35	36.08	35	35	35	35.98	35
Result	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass

Table 63: A summary of the fold regulation values obtained for the *A. racemosa* extract and Sheng Ma extract – values exceeding 2 show significant increased expression of a gene and negative values exceeding 2 show significant decreased expression of a gene compared to the control group

Position	Symbol	APAP	AR 100	AR 20	AR 5	Sheng Ma 100	Sheng Ma 20	Sheng Ma 5
		Fold Regulation	Fold Regulation	Fold Regulation	Fold Regulation	Fold Regulation	Fold Regulation	Fold Regulation
A01	ABCB1	1.026	1.1893	1.4189	1.0464	-1.0725	-1.0041	1.1264
A02	ABCB11	-6.8829	-1.4656	-1.3934	-1.0055	1.0063	-1.1376	-1.4389
A03	ABCB4	-2.4335	-1.028	-1.0013	-1.0604	1.0109	1.0773	1.0461
A04	ABCC2	-5.6687	-1.2583	-1.0682	-1.0801	1.0227	1.1256	-1.0485
A05	ABCC3	-1.9359	-1.1863	-1.0511	1.1882	-1.0336	-1.0371	-1.0754
A06	ALDOA	-2.3506	-1.1539	-1.1214	1.0225	-1.0217	-1.1297	-1.1185
A07	APEX1	-1.1119	-1.0841	-1.2018	-1.0927	-1.131	1.031	1.0012
A08	ASAH1	2.1096	-1.3255	-1.213	-1.2994	-1.1284	-1.0371	-1.1211
A09	ATP8B1	4.6816	-1.4555	-1.2074	-1.0242	-1.2725	-1.1914	-1.0245
A10	AVPR1A	-1.1511	-1.4355	-1.5895	-1.771	-1.455	1.0334	1.0632
A11	BHMT	2.1992	-1.2085	-1.0535	-1.2152	-2.0529	1.123	-1.1796
A12	BTG2	-2.4335	-1.3332	-1.0036	1.0537	-1.0408	-1.0371	-1.0412
B01	CA3	-1.0231	1.0282	-1.1008	-1.0776	-1.6986	1.0999	1.2879
B02	CASP3	1.4211	-1.1987	-1.3934	-1.7628	-1.1001	-1.1014	-1.0705
B03	CCNG1	-1.3407	-1.3533	-1.2413	-1.3831	-1.232	-1.1297	-1.1823
B04	CD36	-1.4671	-2.8909	-1.8216	-3.5175	-2.034	-1.2769	-1.5386
B05	CD68	-2.2238	1.237	-1.013	1.1319	1.2218	1.2606	1.2157
B06	CDC14B	1.1543	-1.1946	-1.1582	-1.3206	-1.1873	-1.0252	-1.1447
B07	CDKN1A	1.3632	-1.1754	-1.1136	-1.147	-1.1389	-1.0371	-1.0151
B08	COL4A1	2.8619	-1.2612	1.1055	1.5606	-1.1155	-1.7005	-1.0631
B09	CRYL1	2.7453	-1.4222	-1.0857	1.0513	-1.3829	-1.0444	-1.0293
B10	CXCL12	1.026	1.1893	1.4189	1.0464	-1.0725	-1.0041	1.1264
B11	CYP1A2	1.0331	1.0498	1.0056	1.1398	10.2603	1.6943	1.1007
B12	DDIT4L	-2.0463	-2.1089	1.4288	-1.3391	-1.3389	1.3263	-1.142
C01	DDX39A	-2.1038	-1.015	-1.1034	-1.0902	-1.1655	1.0051	-1.0606
C02	DNAJB11	1.092	-1.1341	-1.1663	-1.1875	-1.2066	-1.1402	-1.185
C03	DNAJC3	-1.0519	-1.2715	-1.1136	-1.1338	-1.2066	-1.0158	-1.1368
C04	FABP1	-66.8572	-1.6413	-1.0983	1.0084	-2.9711	-1.2419	-1.2212

C05	FADS1	-2.6445	-1.0448	-1.0832	-1.0604	-1.0504	-1.0371	-1.0245
C06	EMC9	-1.0966	1.039	-1.0295	-1.0055	-1.08	1.0263	1.0583
C07	FASN	-1.4773	-1.4792	-1.0807	1.6156	-1.2784	-1.2739	-1.2819
C08	FMO1	-2.7189	-1.1069	-1.2822	-1.4319	1.0086	1.7951	1.3272
C09	TIMM10B	1.195	-1.0569	-1.0319	-1.0433	-1.0336	-1.0064	1.0461
C10	GADD45A	3.2198	-1.0606	-1.0438	1.0908	-1.003	1.0748	1.2556
C11	GCLC	2.5973	-1.3978	1.2641	-1.7345	1.1453	1.448	1.1395
C12	GSR	1.4409	-1.5983	-1.0658	-1.364	1.091	-1.1219	1.0317
D01	HAO2	-20.4356	-2.0347	-1.3213	-1.182	-2.5158	-1.3067	-1.3118
D02	HMOX1	1.5988	1.188	1.0753	1.0709	1.1011	1.0334	1.0981
D03	HPN	-11.1813	1.3	1.1794	1.2618	-1.0077	-1.0134	1.1342
D04	HYOU1	2.6335	-1.2482	-1.1239	1.0984	-1.2263	-1.2024	-1.1988
D05	ICAM1	-2.178	1.027	-1.0584	-1.029	1.236	1.0382	1.0152
D06	IGFALS	1.7496	1.1278	1.3994	2.6984	1.219	1.123	1.4258
D07	IL6ST	1.4409	-1.3409	-1.2733	-1.364	-1.1232	-1.0565	-1.1159
D08	IPO4	1.6099	-1.0618	1.1904	1.5787	1.1856	-1.03	1.0755
D09	FAM214A	-1.5294	-1.1341	-1.1423	-1.2727	-1.1468	1.0798	1.0012
D10	KRT18	-2.0181	1.0919	1.0556	1.0537	1.1506	1.0624	1.0755
D11	KRT8	-1.8063	-1.1686	-1.2158	1.1398	-1.003	-1.0419	1.0058
D12	L2HGDH	2.7076	-1.4371	-1.0153	-1.2322	-1.2377	-1.0252	-1.2016
E01	LGR5	-1.8961	-2.2681	1.0244	-1.4187	-1.5995	-1.4532	-1.3707
E02	LPL	-1.1591	-1.1018	-1.1798	-1.1711	-1.0651	-1.0444	-1.098
E03	LSS	-1.9766	-1.3409	-1.0487	1.3903	-1.3389	-1.4135	-1.2212
E04	MAOB	-2.0322	-1.4707	-1.1188	1.0131	-1.3327	-1.1615	-1.1741
E05	MAP3K6	-1.7814	-1.2424	-1.0366	1.3093	-1.1001	-1.0712	-1.068
E06	MBL2	-1.7814	-1.3754	-1.1344	-1.2815	-1.6107	-1.2164	-1.1315
E07	MCM10	-1.6737	-1.138	-1.3001	-1.1105	1.3186	1.1178	1.0175
E08	MLXIPL	2.4742	1.0894	1.3737	2.0592	-1.0577	1.0145	1.1527
E09	MRPS18B	1.077	1.0013	1.022	-1.1417	1.0986	1.1816	1.1608
E10	NQO1	-3.3012	-1.3818	-1.3031	-1.6035	1.0086	1.0649	-1.2016
E11	NUS1	1.0189	-1.0791	-1.1449	-1.3863	-1.0312	1.0674	-1.046
E12	OSMR	-2.6263	-1.0643	-1.0807	-1.4858	1.0539	1.0005	-1.196
F01	SLC51A	-38.9354	-1.0557	-1.0511	-1.2465	2.625	1.0773	-1.1714
F02	PDYN	1.026	1.1893	1.4189	1.3461	1.8734	1.0239	1.1264
F03	PLA2G12A	2.9424	-1.2183	-1.1265	-1.1985	-1.1736	1.043	-1.1473

F04	PPARA	1.6099	-1.2907	-1.1529	-1.0314	-1.2377	-1.0492	-1.1031
F05	PSME3	1.3077	-1.1673	-1.0983	1.0392	-1.1655	-1.0324	-1.0436
F06	PYGL	-2.4846	-1.2759	-1.2674	-1.1902	-1.0577	-1.0762	-1.098
F07	RB1	-1.2596	-1.4438	-1.4128	-1.4024	-1.2666	-1.1859	-1.2071
F08	RDX	1.195	-1.2877	-1.2558	-1.4824	-1.0775	-1.0614	-1.1031
F09	RHBG	2.2926	-1.7409	-1.2733	2.0076	-1.9022	-1.3434	-1.719
F10	S100A8	-4.8333	1.6116	1.2409	1.0108	1.7806	1.4547	1.3457
F11	SCD	-2.8739	-1.2367	-1.2046	-1.3176	-1.1495	-1.1323	-1.0754
F12	SERPINA3	-5.3258	1.2185	1.1659	-1.0433	1.1347	1.0334	1.1661
G01	SERPINE1	-1.857	-1.1288	-1.2102	-1.0653	1.4035	-1.0787	-1.1159
G02	SKIL	1.2544	-1.7031	-1.4194	-1.4319	-1.2992	-1.2419	-1.4158
G03	SLC17A3	-4.0084	-1.098	-1.0106	-1.1182	1.0039	1.1127	-1.0412
G04	SLC2A3	1.026	1.1123	1.3269	-1.0219	-1.1468	-1.0737	1.0534
G05	SLC39A6	2.0951	-1.2656	-1.2911	-1.4552	-1.0775	-1.1193	-1.1159
G06	SREBF1	-4.0926	-1.4388	1.0508	1.7437	-1.1103	-1.1695	-1.15
G07	TAGLN	-1.6621	-1.1301	1.0803	1.5285	-3.9659	1.1981	-1.0779
G08	THRSP	-1.4671	-3.2188	-1.2529	1.1664	-4.1631	-1.2622	-1.1185
G09	TMEM2	1.3538	-1.0741	-1.1609	-1.3609	1.049	1.0028	-1.0436
G10	TXNRD1	3.8825	-1.5456	-1.3152	-1.2322	-1.0553	-1.208	-1.1905
G11	WIPI1	1.7987	-1.2892	-1.2529	-1.204	-1.2962	-1.1297	-1.2127
G12	YRDC	2.6518	-1.046	1.0483	1.1086	1.0514	1.0215	1.0981
H01	ACTB	-2.163	-1.0484	1.0291	1.0038	1.1088	1.1127	1.1395
H02	B2M	-1.0966	-1.0692	1.0033	-1.1312	-1.0976	1.0098	1.0128
H03	GAPDH	-4.541	1.1356	1.2126	1.3032	1.1829	1.0098	1.0981
H04	HPRT1	1.1305	-1.2141	-1.2018	-1.2639	-1.2235	-1.1271	-1.166
H05	RPLP0	1.4015	1.0259	-1.0414	-1.0826	-1.0147	1.0798	1.027
H06	HGDC	1.026	1.1893	1.4189	1.0464	-1.0725	-1.0041	1.1264
H07	RTC	1.0118	1.0024	1.4454	1.6008	1.1011	1.2261	1.2213
H08	RTC	1.0845	1.0806	1.3929	1.6045	1.1088	1.2782	1.2326
H09	RTC	1.077	1.1757	1.4929	1.7117	1.1037	1.308	1.2969
H10	PPC	1.1463	1.0223	1.1605	-1.0851	1.0063	1.0999	1.1342
H11	PPC	-1.1119	-1.052	1.0654	-1.1054	-1.075	1.0823	1.0558
H12	PPC	-1.074	1.0176	-1.2733	-1.1182	-1.0194	1.1178	1.1688

As mentioned in section 5.3.2. Treatment and gene expression analysis of HepaRG™ cells, the C_t values are converted to fold change and fold regulation values through several calculation stages.

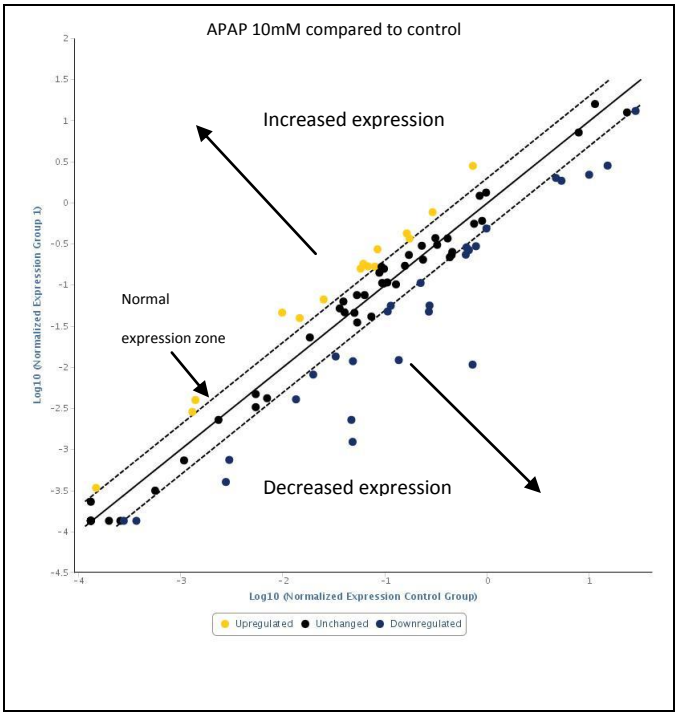


Figure 69: A Scatter plot of gene activity in HepRG™ cells treated with acetaminophen – increased expression of genes shown as yellow dots, decreased expression of genes shown as blue dots and unaffected genes are displayed as black dots within the dotted line zone.

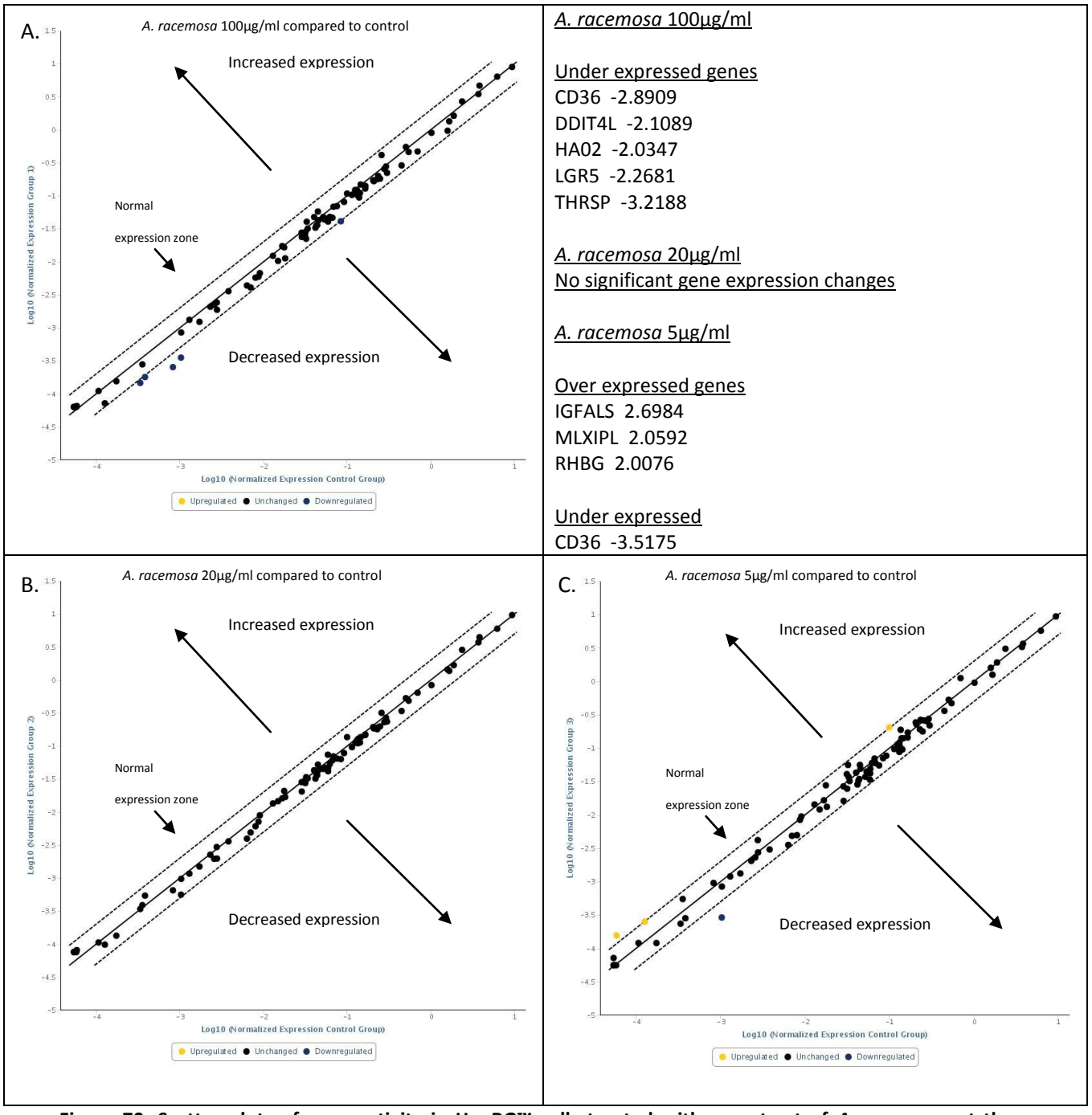
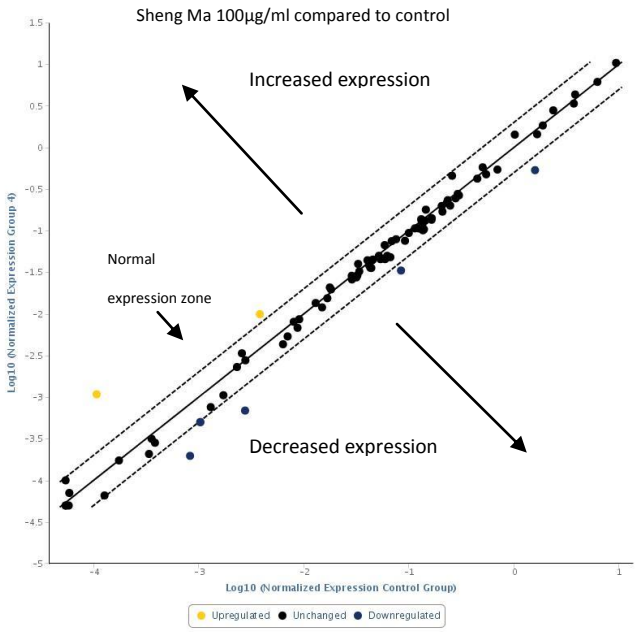


Figure 70: Scatter plots of gene activity in HepRG™ cells treated with an extract of *A. racemosa* at three different concentrations. A. *A. racemosa* 100µg/ml, B. *A. racemosa* 20µg/ml, and C. *A. racemosa* 5µg/ml, – increased expression of genes shown as yellow dots, decreased expression of genes shown as blue dots and unaffected genes shown as black dots within the dotted line zone.



Sheng Ma "*A. dahurica*" 100µg/ml

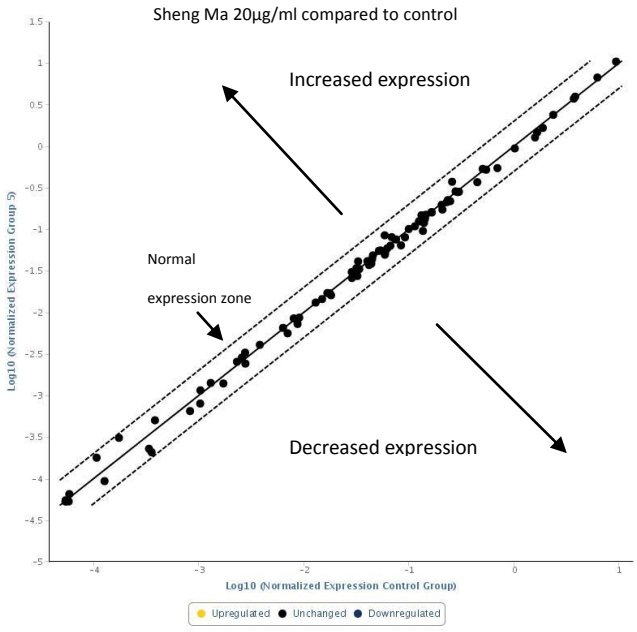
Under expressed genes
BHMT -2.0529
CD36 -2.034
FABP1 -2.9711
HA02 -2.5158
TAGLN -3.9659
THRSP -4.1631

Over expressed genes

CYP1A2 10.2603
SLC51A 2.625

Sheng Ma "*A. dahurica*" 20µg/ml
No Significant Gene Expression changes

Sheng Ma "*A. dahurica*" 5µg/ml
No Significant Gene Expression changes



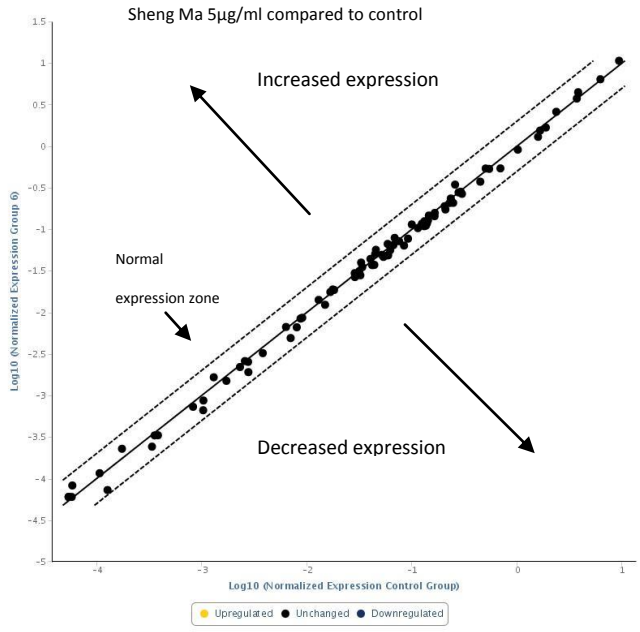


Figure 71: Scatter plots of gene activity in HepRG™ cells treated with an extract of *A. racemosa* at three different concentrations. A. Sheng Ma 100µg/ml, B. Sheng Ma 20µg/ml, and C. Sheng Ma 5µg/ml, – increased expression of genes shown as yellow dots, decreased expression of genes shown as blue dots and unaffected genes shown as black dots within the dotted line zone.

The Log^{10} of the fold regulation is displayed in the scatter plots as under expressed genes are made negative from the calculation and allows for a meaningful visual plot.

The results for *Actaea racemosa* treated cells show decreases in expression of genes at 100µg/ml. The gene expression changes of all three concentrations are displayed in Figure 72. There were no over expressed genes shown. CD36 (thrombospondin receptor), DDIT4L (DNA-damage-inducible transcript 4-like), HA02 (Hydroxyacid oxidase 2 (long chain)), LGR5 (Leucine-rich repeat containing G protein-coupled receptor 5) and THRSP (Thyroid hormone responsive) are all under expressed (i.e. more than a 2 fold change in expression) compared to the control. Increased expression of CD36 is seen in steatosis but the opposite was seen in the experiment. Increased expression of DDIT4L is seen in hepatotoxicity but a decreased expression was seen. HA02 and THRSP are seen as down regulated in hepatotoxicity which is what was seen in this experiment but HA02 was marginal. LGR5 is decreased in expression in necrosis as was seen in the experiment. At the lower concentration of 5µg/ml there are a few marginal over expressions seen in IGFALS (Insulin-like growth factor binding protein, acid labile subunit), and marginal over expression seen in MLXIPL (MLX interacting protein-like) and RHBG (Rh family, B glycoprotein (gene/pseudogene)). IGFALS, MLXIPL and RHBG are under expressed in hepatotoxicity but they were over expressed in these tests. A marginal under expression was shown in CD36 which is over expressed in steatosis. The only significant changes are perhaps THRSP or HAO2 which are down regulated as seen in hepatotoxicity and LGR5 which is shown in necrosis according to the manufacturers of the qPCR array (Qiagen Inc.,CA).

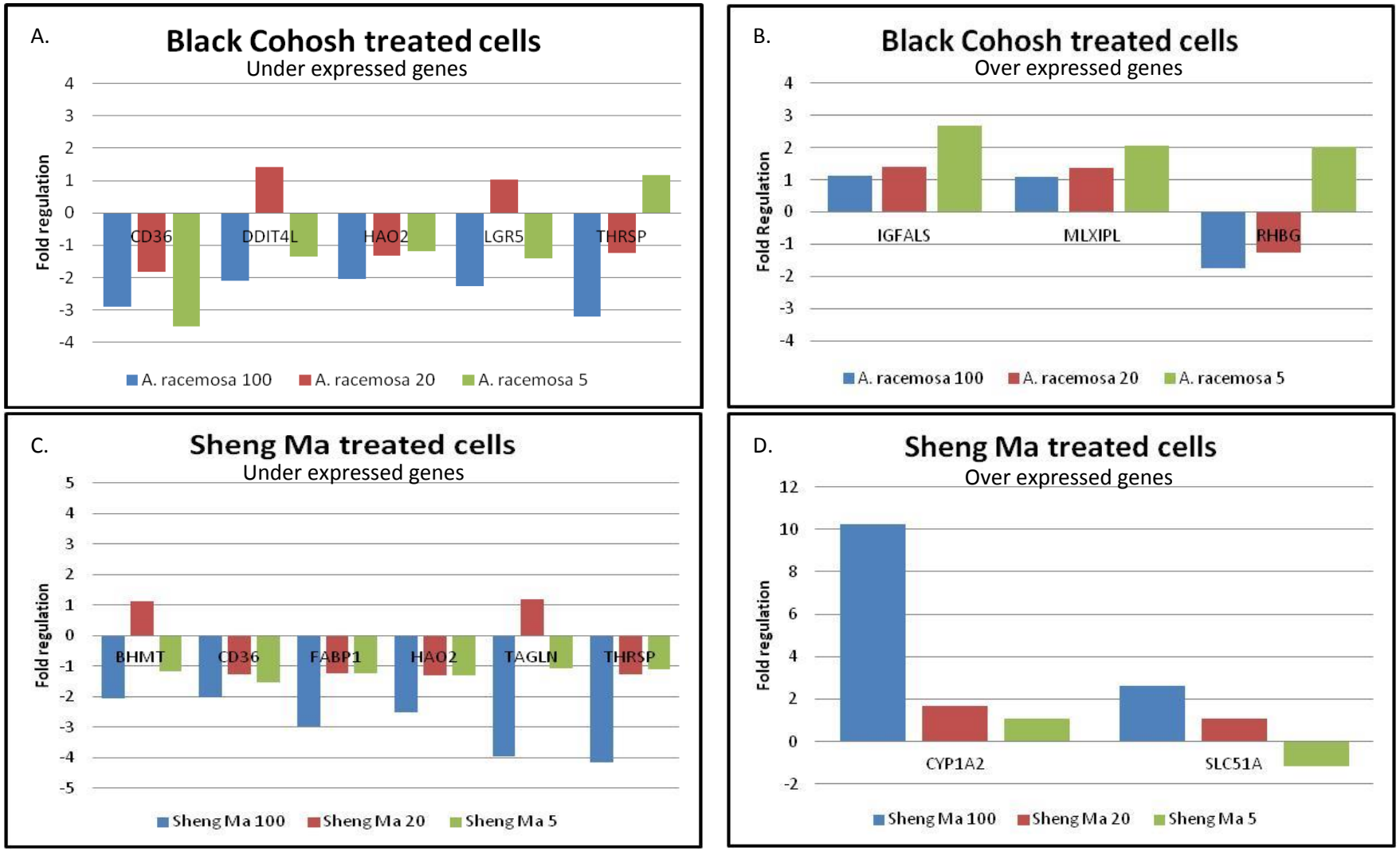


Figure 72: Bar charts to show gene expression changes in cells treated with Black Cohosh and Sheng Ma extracts. A. Under expressed genes after treatment with Black Cohosh extract. B. Over expressed genes after treatment with Black Cohosh extract. C. Under expressed genes after treatment with Sheng Ma extract. D. Over expressed genes after treatment with Sheng Ma extract.

As for the Sheng Ma extract, several changes are seen in gene expression. Figure 72c and d display the changes of expression in chart form. At 100µg/ml CYP1A2 (Cytochrome P450, family 1, subfamily A, polypeptide 2) and SLC51A (Organic solute transporter alpha) were seen to be over expressed. CYP1A2 is usually seen to be down regulated in hepatotoxicity but this change is still interesting as it is a significant increase. SLC51A has an involvement in cholestasis. THRSP is under expressed as seen in hepatotoxicity, TAGLN (Transgelin) is under expressed as seen in phospholipidosis, FABP1 (Fatty acid binding protein 1, liver) is under expressed but usually over expressed in phospholipidosis, and HAO2 is under expressed in hepatotoxicity. There was also decreased expression of CD36 which is usually increased in steatosis and BHMT (Betaine--homocysteine S-methyltransferase) was marginally under expressed as seen in hepatotoxicity. There were no changes seen in the other two concentrations. These changes are very interesting and show that at certain doses of the Sheng Ma extract induces changes in genes associated with phospholipidosis, cholestasis and hepatotoxicity. It could be said more firmly if the Sheng Ma extract induced these disease pathways if more genes included in the same pathway were found to be altered in line with what would be expected. The grouping of the genes expected to change for each pathway is outlined in Table 51.

5.3.3 Lactic dehydrogenase (LDH) assay

For this assay the treated HepaRG™ cells and treated HepG2 cells were analysed. The media was assayed for this test as the released LDH was the indication of toxicity. Various concentrations and all three extracts were utilised. Each differing sample was analysed in triplicate and the average result is shown. The results are summarised in Table 64 and a chart of the results is displayed in Figure 73.

Table 64: The spectrophotometry values obtained when measuring released LDH in treated HepG2 and HepaRG™ cultured cells.

Sample	Average reading at 490nm	
	HepaRG™	HepG2
Control (DMSO)	0.368	0.366
A. racemosa 100µg/ml	0.385	0.416
A. racemosa 20µg/ml	0.369	0.396
A. racemosa 5µg/ml	0.372	0.387
Sheng Ma *A. dahurica* 100µg/ml	0.372	0.523
Sheng Ma *A. dahurica* 20µg/ml	0.356	0.447
Sheng Ma *A. dahurica* 5µg/ml	0.368	0.363
Sheng Ma *A. cimicifuga* 100µg/ml	0.376	0.946
Sheng Ma *A. cimicifuga* 20µg/ml	0.362	0.364
Sheng Ma *A. cimicifuga* 5µg/ml	0.326	0.358

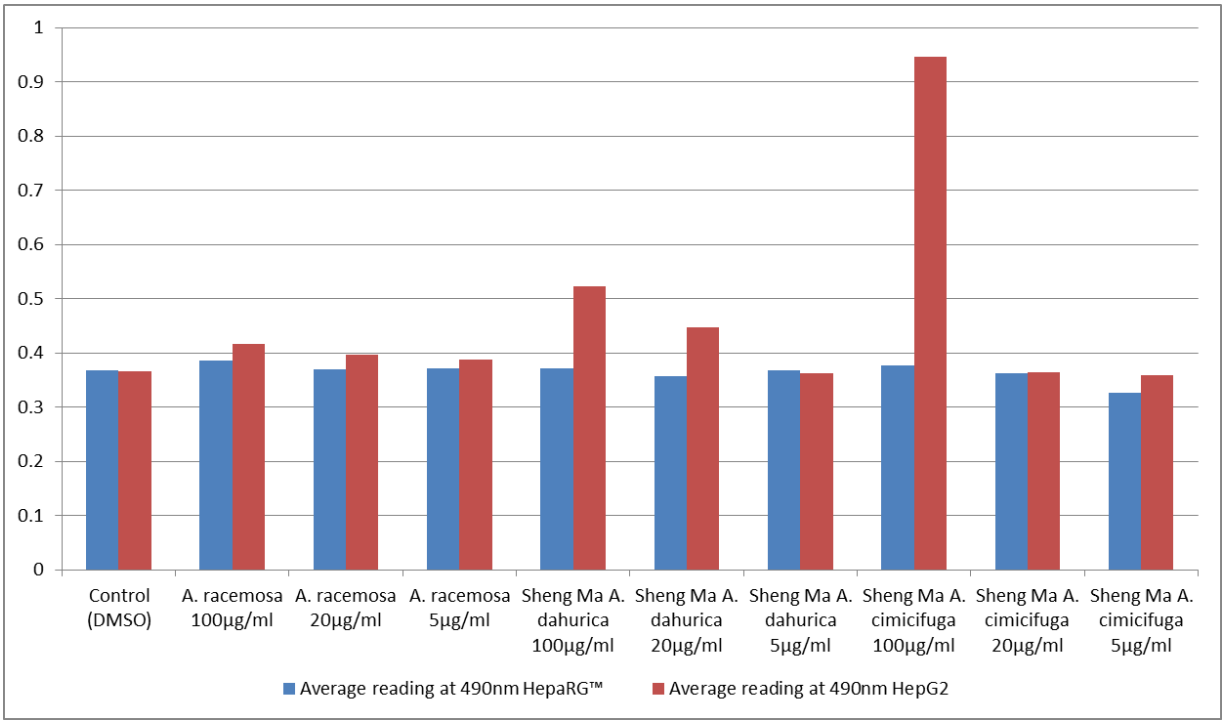


Figure 73: A bar chart to show the spectrophotometry readings of each cell type after treatment with the *A. racemosa* and Sheng Ma extracts. The HepaRG™ and HepG2 cells are shown side by side for comparison.

The HepaRG™ cells when treated with *Actaea* extracts did not appear to release much LDH or have any significant differences between the ranges of concentrations. The HepG2™ cells however

appeared to be much more sensitive to the treatment of the extracts. The amount of LDH release dropped as the concentration was decreased.

5.3.4 MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

HepG2 cells were seeded into a 24 well plate and treated with various concentrations of *Actaea* extracts. This analysis was not available for the HepaRG™ cells at the time of experimentation and the cells are required for this test rather than the media as in the LDH assay. Each test was completed in triplicate and the average readings are shown. Results are summarised in Table 65 and shown in Figure 74. The number of viable cells is directly related to how intense the colour reaction is as healthy cells are able to reduce the MTT reagent to formazan.

Table 65: Results of the MTT assay - HepG2 cells treated with *Actaea* extracts at three concentrations – calculated viability shown.

Sample	Average reading at 490nm	% viability
Cells only	1.23	100.00
Sheng Ma *A. dahurica* 100µg/ml	1.14	92.68
Sheng Ma *A. dahurica* 20µg/ml	1.07	86.99
Sheng Ma *A. dahurica* 5µg/ml	1.09	88.60
Sheng Ma *A. cimicifuga* 100µg/ml	0.73	59.35
Sheng Ma *A. cimicifuga* 20µg/ml	0.81	65.85
Sheng Ma *A. cimicifuga* 5µg/ml	0.91	73.98
A. racemosa 100µg/ml	0.97	78.86
A. racemosa 20µg/ml	1.01	82.11
A. racemosa 5µg/ml	1.05	85.37
DMSO control	1.30	105.69

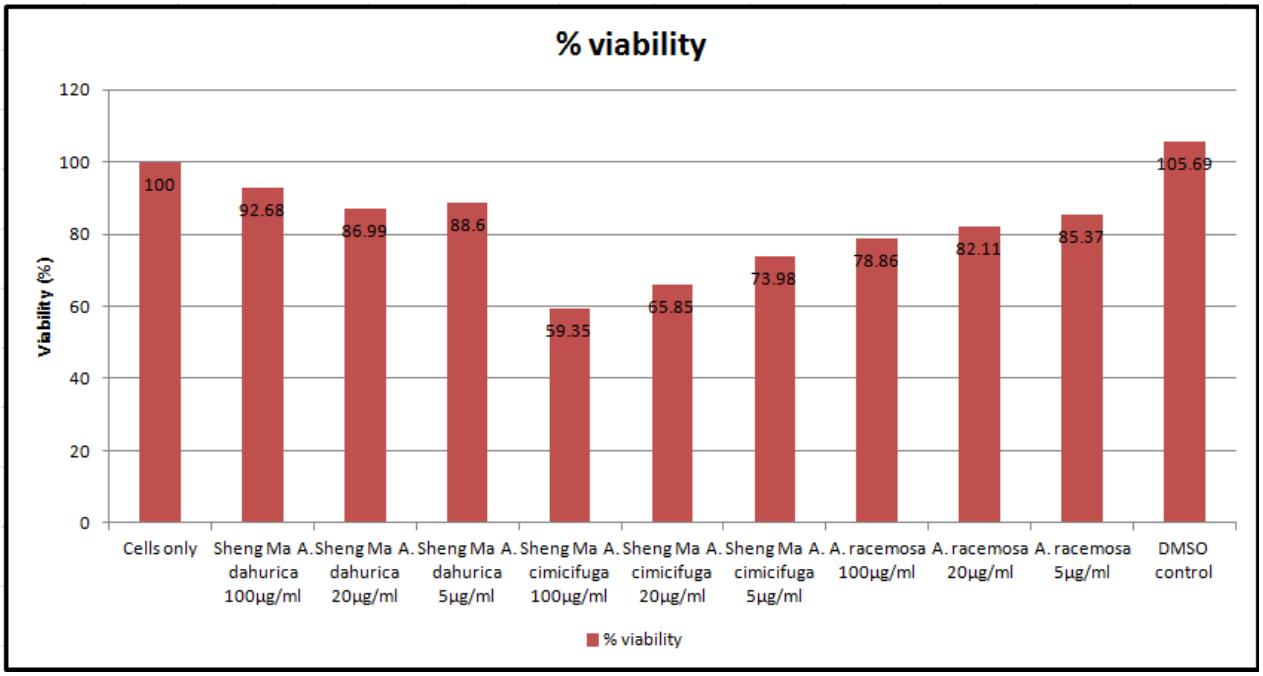


Figure 74: A chart to show the calculated cell viability of the cells after treatment with the *A. racemosa* and Sheng Ma extracts.

It was shown in the MTT assay that the extracts had an effect on viability of the cells. The viability increased as the concentration of extracts decreased.

5.4 Conclusions and further work

The *Actaea dahurica* and *Actaea cimicifuga* samples that were obtained appear to be a mixture of both species in both materials. Morphological examination was unable to help with identification due to the nature of the materials, i.e. only dried roots were present for study and they had been processed by cutting making microscopy difficult. The *Actaea racemosa* material was grown under the control of Schwabe GmbH so had the advantage of flowering plants being available for identification. The other two materials could not be identified conclusively using macro or microscopic visual methods. The rhizomes had been processed into slices and this hindered the identification using these methodologies. The HPTLC results showed also a mixture of the two species being present in both samples. Markers for both *Actaea cimicifuga* were seen in both samples. The results from the DNA analysis support this. The materials were sourced from China. The Chinese Pharmacopoeia method for identifying these species (based on detection of ferulic and

Isoferulic acid using TLC) does not discriminate between any of the following; *Actaea cimicifuga, Actaea dahurica* or *Actaea heracleifolia*. The reason for this is that all of these species are acceptable to be used in the Chinese medicine Sheng Ma interchangeably. This Chinese Pharmacopoeia identification method is presumably used by the company that the materials were sourced from, as they supply Chinese medicinal herbs, so one could assume that identification was made during harvesting and just affirmed to be one of the four desired species later on using Chinese Pharmacopoeia monograph methods. The *Actaea racemosa* material was shown to be the true identity using all three methods.

The materials labelled as *Actaea racemosa, Actaea cimicifuga* and *Actaea dahurica* were all used in the pilot qPCR gene expression study. Due to the lengthy process of cell treatment through to gene expression, the pilot study was kept minimal. Once it was shown that all the steps could be successfully completed the main experiment was carried out. Due to the *Actaea dahurica* and *Actaea cimicifuga* samples both appearing to be a mixture of the two species, only one of these was included in the rest of the study and was termed as Sheng Ma. This maximised the variation of concentrations that could be used and allowed biological triplicates to be utilised: the qPCR array data showed that *Actaea racemosa* was not harmful. There were some gene expression differences present but none that were significant. The Sheng Ma *Actaea dahurica* extract did seem to induce gene expression changes linked with hepatotoxicity, cholestasis and phospholipidosis. An interesting finding is that the Sheng Ma species extract was found to increase expression of CYP1A2. There have been many substances proven to be inducers of CYP1A2 such as coffee, cigarette smoke, cruciferous vegetables and various prescription drugs including Omeprazole and Nelfinavir (Zanger and Schwab, 2013), but there has not been previous evidence of Sheng Ma as an inducer. There were various genes changes that were seen related to hepatotoxicity and phospholipidosis. THRSP, BHMT and HAO2 were seen to be under expressed which can be seen in hepatotoxicity. TAGLN (Transgelin) is under expressed in phospholipidosis which was seen in the experiment. SLC51A (Organic solute transporter alpha) was seen to be over expressed and this is seen in cholestasis. Increased expression

of this gene has been found in patients with primary biliary cirrhosis (Ballatori et al., 2013). BHMT has been shown to have a role in protecting the liver and has been found to be decreased in expression in diseased liver (Feng et al., 2011). HAO2 has a role in fatty acid metabolism and is impaired in hepatotoxicity (Mattu et al., 2016).TAGLN is down regulated in phospholipidosis as demonstrated by Nioi et al. It was found to become under expressed when treating HepG2 cells with known phospholipidosis inducing drugs. The function of this gene is not understood (Nioi et al., 2007).

The changes seen in the cells treated with the *Actaea racemosa* extract where just as numerous but the majority of the changes were the opposite of what would be expected in liver disease and liver injury. There were some slight changes seen in two genes associated with hepatotoxicity compared to the control; THRSP and HAO2, as was also seen in Sheng Ma, but both genes were not increased as much as Sheng Ma. LGR5 (Leucine-rich repeat containing G protein-coupled receptor 5) was slightly under expressed and this gene is related to necrosis when under expressed according to the manufacturer of the qPCR array. A review of some current literature (Huch and Dolle, 2016, Khan et al., 2017, Khan et al., 2015) has shown that the expression of LGR5 is in fact increased in liver injury as the function of the gene has a role in regeneration following injury to the liver.

The reliability of the control is very important as any changes in gene expression are a result of comparison. The use of proven housekeeping genes is also fundamental to the study as they are used for normalisation. In the final gene expression study each test sample was analysed in biological triplicates. Controls for detection of genomic DNA contamination, reproducibility of the arrays and reverse transcription were built into each array and so a good level of confidence can be given to the results obtained. The gene changes that were detected for the treatment with the Sheng Ma extract were not large in comparison to acetaminophen for example but they were still significant. It can't be concluded from the study that Sheng Ma i.e. Asian *Actaea* causes hepatotoxicity but there are signs of the extract causing changes within the cells at a molecular level. A study for a longer period of time needs to be conducted.

The LDH results were interesting as it gave the opportunity to compare the effects of two different liver cell lines. The HepG2 cells seemed to be much more sensitive to the effects of the extracts, particularly with Sheng Ma *Actaea cimicifuga*. The MTT assay was also carried out. The MTT assay allows the measurement of conversion of the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. MTT is soluble and formazan is insoluble within the media. This allows the formazan to be precipitated. The precipitate is then dissolved in another solvent (in this case acidified isopropanol) and intensity of the colour measured. The more dissolved formazan present the more active the cells are, as they are able to carry out the conversion. This shows viability of the cells. The HepG2 cells were used in this experiment. All three of the *Actaea* extracts were used as treatments. All of the extracts had an effect on the cells at the highest concentrations. The effect of the extracts reduced as the concentrations reduced. The *Actaea cimicifuga* Sheng Ma extract had the largest effect on the cells, second was *Actaea dahurica* Sheng Ma extract and finally *Actaea racemosa* had the least effect. These results are all interesting. It shows that Sheng Ma species are potentially more harmful than *Actaea racemosa.* As mentioned in the introduction it has been found that *Actaea cimicifuga* is cytotoxic to cancer cell lines (HepG2 cells IC_{50} value was 21 µg/mL) (Tian et al., 2007, Chen et al., 2014a), and as HepG2 cells were used for this treatment it is perhaps not very surprising that a reduction in cell viability was seen. The important point would be to find out if the same is seen in primary human hepatocytes.

Overall the Sheng Ma extracts showed more evidence of causing ill effects to the liver cells that they were used to treat. The *Actaea racemosa* extract showed a slight under expression in two genes associated with hepatotoxicity but they did not show such an effect with the LDH or MTT assays. On the other hand the Sheng Ma species extracts showed gene expression changes in several liver disease pathways and triggered an over expression of CYP1A2 which although is not a marker of hepatotoxicity, is an interesting change as it has not been seen in any published literature to date. The Sheng Ma species extracts also caused the cells to produce more LDH and showed the least viability in the MTT assay.

6 Overall Conclusions

6.1 The importance of prior quality control of reference databases

The key theme overall in this thesis is the reliable identification of raw medicinal plant materials. This is not just important for the manufacture of herbal medicines, but it is also fundamental in developing the use of barcode sequences for identification means. In order for a plant sample to be used as a reference, there have been extensive conditions set out in the literature by Smillie and Khan 2010. These include full documentation of the site of harvest, photos, sampling and inclusion into a formally recognised herbarium. There also needs to be a full morphological characterisation of the above ground plants and of the part of the plant that is used in the herbal medicine, e.g. flowers, leaves, stem, root etc. This rarely occurs and as a consequence the wrong plant species have been used for barcoding (Smillie and Khan, 2010). This means that some herbal medicines will be made with the incorrect species and also that identification methods become based on the wrong plant species completely mixing up the resulting identification. Using the wrong material in production of herbal medicines has had detrimental effects (Tankeu et al., 2016). The available literature supports the safety and efficacy of authentic Black Cohosh products containing only the species *Actaea racemosa* (Nappi, 2005, Osmers et al., 2005, Wuttke et al., 2006, Molla et al., 2011). Where possible, products associated with adverse reactions have been tested, and have been found to be substituted with Asian *Actaea* species known as Sheng Ma in Chinese medicine(Jordan et al., 2010). Clinical trials that have shown poor efficacy have been linked to products that are not registered and therefore not as robustly tested (Beer and Neff 2013). There has not been a great deal of accessible research into the safety and efficacy of Sheng Ma products. Sheng Ma products are intended for use in Chinese medicine for skin disorders.

When the original PlantID assay for Black Cohosh was designed, there were a small number of ITS sequences of unknown sequence quality available. With the addition of new sequence accessions to

the databases, there is the potential to overcome this problem by looking at trends rather than individual sequences. Several *Actaea racemosa* sequences that all cluster together give more confidence than a single sequence. This is therefore an iterative process – as more sequences accumulate, the tests can and should be re-evaluated. The available DNA sequence data for the nrITS region of the included species was re-assessed. There had to be a high level of confidence in the species identification assigned to the sequence data in order to be able to use it for classification of the test materials. An analysis was carried out based on the similarity of the published DNA sequences and the output for this was represented as a tree diagram. Sequences that were similar were clustered together into branches. The tree was then studied; any sequence accessions that did not match the other entries in the branch that they were assigned to, were not used for identifying materials. Once the sequence data had been assessed it could be used to assign identification to any sequenced samples. This confirmed or sometimes contradicted the species identification that had been assigned to the sequence accession.

6.2 Development of DNA based identification tests – choice and development of suitable assays

The first DNA based identification test was a one tube assay capable of detecting five different species. The assay was developed with reference materials from vouchered herbarium samples which were also positively identified using genomic sequencing. A species specific primer mix was used in a multiplex reaction and showed that when all five target species were present they could all be detected. The main limitation to the assay is the time it takes to develop and optimise it. Once the assay has been developed though, it is a great tool for identification and is easy to perform and gives a solid conclusion. A mixture of singleplex reactions can be run alongside the test reactions to give something to compare to, so that it can be easily seen if a species is present in the test sample or not. The assay requires only the basic tools that a molecular biology lab is equipped with; a PCR machine and gel electrophoresis equipment. This assay would be particularly useful for assessing polyherbal

formulations such as those seen in Chinese and Ayurvedic medicine. The assay design is not limited to identification of Black Cohosh as it has also been developed for St John's Wort. The second of the DNA based identification tests to be developed was the qPCR assays. The same species-specific primers developed for the PlantID assay were utilised. Multiplex qPCR has two main approaches; primers labelled with different fluorescent markers or the use of high resolution melting for assessment of the resulting amplicons. The differently labelled primer detection is restricted to the number that the qPCR machine is able to differentiate between. This number does not usually exceed five and can be financially demanding not to mention difficult technically to develop. High resolution melting is also limited by amplicon size and GC content of the primers. This was shown in this work not to be feasible using the species-specific primers that have been developed. Only two variants were detected from five when attempted using the PlantID assay. It was decided to develop individual species assays using qPCR. This would require more individual reactions which were a downside compared to the PlantID assay but it would not be restricted by the collective annealing temperature required for specificity in the multiplex reaction. Most qPCR machines now have the ability to apply a temperature gradient across the block which opened up the possibility of running reactions with different annealing temperature requirements together. This means that the qPCR assays would not necessarily take longer to complete compared to the plantID assay. The qPCR assays also did not require a separate diagnostic agarose gel and results were available immediately after the reaction was complete. The qPCR assays were developed for *Actaea racemosa, Actaea dahurica* and *Actaea cimicifuga.* The reason these three species were chosen is that they are the most relevant of all the species included in this work. *Actaea racemosa* of course as it is the authentic species for Black Cohosh preparations and then the other two as they have been found to most commonly be detected as adulterants in tested Black Cohosh products. It would have been ideal to include *Actaea dahurica* in the PlantID assay but the required annealing temperature to maintain specificity was too high. The qPCR assay was validated with DNA extracted from samples that had

their identification verified with sequencing. In each tested material the results were consistent with the sequencing results.

6.3 Trialling the developed assay qPCR with genuine commercial products

More than 30 commercial samples were tested using the qPCR assays. The results of this testing were often confirmed with sequencing or a separate chemical analysis completed by Sharaf et al., 2016. This chemical test was mass spectroscopy based and results were visualised using principle component analysis (PCA). It appeared that the reference material used to develop the chemical analysis for *Actaea dahurica* and *Actaea cimicifuga* were identified incorrectly and therefore gave the opposite results of the qPCR assay. These are the kinds of issues that prove how important the reference material of an identification technique is in terms of ensuring that it is of the correct species. Overall the qPCR assays were easy to perform and interpret. Of the 34 commercial products tested 32 were successfully analysed. Five of the products from the UK market had undeclared species detected and 6 of the products from the US market had undeclared species detected. These undeclared species were Asian *Actaea* and demonstrate that current QC testing regimes are either not sensitive enough, or that suppliers are deliberately adulterating or substituting for Asian species.

There are other DNA based assays that have been developed for authentication of Black Cohosh but they have so far been based around restriction enzyme digestions (Xu et al., 2002, Masada-Atsumi et al., 2014, Zerega et al., 2002). These tests are not very reproducible. They are also time consuming to carry out as they all require a diagnostic gel. This can be overcome by using qPCR as utilised in the qPCR assays in this work.

There has not yet been a validated qPCR assay such as the one in this work published. The qPCR assay in this work was validated by using it to test samples with known identities from sequencing efforts. The work of Baker et al., 2012 was a validated study carried out on many samples but the researchers only went as far as to sequence the barcoding region of the included samples. The study was a good contribution to the research community but the qPCR assay in this work goes a step

further and cuts out the need to sequence the samples. Sequencing can be relatively expensive compared to a qPCR reaction and is limited to the presence of good quality DNA that is not degradated. The group did use mini-*matK* barcodes to overcome DNA degradation but as shown in Chapter 2 the *matK* region is not ideal for identifying *Actaea* species due to the sequence entries in GenBank clustering together when organised in a DNA tree. This shows that the mini-*matK* region is too similar between different species within the *Actaea* genus to reliable as a means of identification alone. The *matK* region was only sequenced in this work to backup existing results. This work also shows that there are still products available on the market that contains undeclared species. The Traditional Herbal Medicines Directive (Directive 2004/24/EC) was put in place to regulate the products available on the market but there are grey areas in the legislation which allow products to be sold as supplements. In order to sell a product as a supplement there can be no claim to treat disease. The products that are registered as herbal medicines go through a costly process which includes being able to show traditional usage and safety of use. This shows the importance of having assays that are able to detect adulterant species and that are able to do so at a highly sensitive level. None of the THR (Traditional Herbal Registration) products were found to contain undeclared species although the included number was few. Products from the US are regulated as 'dietary supplements' through the Dietary Supplement Health and Education Act (DSHEA) of 1994. The current legislation allows the presence of products on the US market without first demonstrating efficacy or safety and so are not scrutinised as much as THR products on the UK and European market. Although these products are not allowed to be marketed with claims of treating disease, they are permitted to make loose claims about benefiting health (Bent, 2008). These are further reasons of the need for robust tests being available. The qPCR assays in this work could be the answer to this issue. They could also be developed for other *Actaea* species and other non *Actaea* species.

The next aim will be to publish the sequence data generated as part of this work. For some of the *Actaea* species there are limited entries for the ITS region and so this data will be useful for many researchers and a valuable contribution to the Barcode of Life Project.

The conclusion of this work is that raw materials must be authenticated prior to being used and sold in products. The assays developed in this work could contribute to that cause. The British Pharmacopeia are already ahead on this aspect. DNA based authentication is in use and included in recommended protocols. DNA barcoding is moving forward at great speed and will one day be a rapid analysis available to all.

6.4 Assessment of potential hepatotoxicity of *Actaea racemosa* and Asian *Actaea* species

The next issue to be addressed are the claims that Black Cohosh has caused liver injury in isolated case reports. These case reports have been reviewed again and again using different methods. Each time it is found that the link to the use of Black Cohosh is weak (Mahady et al., 2008, Teschke et al., 2011). The patients are often found to be taking other medicines that can affect the liver or they consume alcohol. The cases are also found to be poorly documented and key events such as dechallenge and rechallenge either do not occur or are not recorded (Teschke et al., 2014). Important tests such as measure of liver enzymes are either not taken or not recorded (Teschke et al., 2011). A review of the case reports was carried out by (Teschke et al., 2013) who looked for alternative causes of hepatotoxicity in each instance. It was found that of 96 reports, 52 were due to other causes such as hepatitis infection, cirrhosis or liver injury from comedication to name but a few. Even though the case reports have found to have a poor connection to Black Cohosh usage, it is still required in countries across the globe to include a warning label on Black Cohosh products. As said previously, products indicated in liver injury cases have been tested when available and have been found to contain other undeclared Asian *Actaea* species. This raises the question of whether the presence of Asian *Actaea* species is in fact the cause of liver toxicity in these cases. Clinical trials support the safety and efficacy of Black Cohosh when the products contain only *Actaea racemosa* (Nappi, 2005, Osmers et al., 2005, Wuttke et al., 2006, Molla et al., 2011). There are no clinical trials that include any Sheng Ma species available. Rats have been used as a model to treat with Black

Cohosh and this has shown to be safe (Briese et al., 2007, Mazzanti et al., 2008). Some in vitro studies have been conducted using human hepatocyte cell lines. Huang et al., 2010 treated hepG2 cells with different types of Black Cohosh extracts and did not find any significant cell viability changes using the MTT assay as an assessment. Tian et al., 2005 and 2007 treated HepG2 cells with fractions and isolates from the aerial parts of *A. dahurica* and *A. cimicifuga.* They found that treatment induced apoptosis in the cells. It cannot be known without further testing if ethanol extracts of the roots/rhizomes would induce the same changes however. This is where a gap in the conducted research has been identified. There needs to be in vitro testing of *Actaea racemosa* and Sheng Ma species to determine if any cause hepatotoxicity.

To address the uncertainty of toxic activity of *Actaea racemosa* or Sheng Ma species extracts, a series of cell culture based assays were employed. The first of the assays was a large gene expression study assessing 84 genes associated with hepatotoxicity. Rhizome material for *Actaea racemosa, Actaea dahurica* and *Actaea cimicifuga* were sourced and confirmation of identification was attempted using DNA testing, morphological assessment and HPTLC. The identification of the *Actaea racemosa* material was confirmed with all tests. The identification of the other two materials was much more difficult to confirm and the outcome was that both materials appeared to be a mixture of *Actaea dahurica* and *Actaea cimicifuga.* For this reason only one was used for the final gene expression study. The materials were used to manufacture 60% ethanol extracts and these were used for the treatment of HepaRG™ cultured human liver cells. The results of these tests showed gene expression changes in the cells treated with both the *A. racemosa* extract and the Sheng Ma extract. The Sheng Ma ethanolic extract induced changes associated with hepatotoxicity, cholestasis and phospholipidosis. Unfortunately there were only a few genes from each disease pathway that showed an expression change. It would have been more conclusive if more of the genes associated with each of the disease had the expected changes. An interesting finding is that Sheng Ma was found to increase expression of CYP1A2. There have been many substances proven to be inducers of CYP1A2 such as coffee, cigarette smoke, cruciferous vegetables and various prescription drugs

including Omeprazole and Nelfinavir (Zanger and Schwab, 2013), but there has not previously been evidence of Sheng Ma species as an inducer. The *Actaea racemosa* induced changes which were often the opposite of what would be expected in liver injury. There were some gene expression changes associated with liver injury and although these changes were a significant fold change, (more than 2) in the highest concentration, they were few in number compared to the Sheng Ma results.

The MTT assay was performed following extract treatment on the HepG2 cell line. This has been performed on these cells following treatment with *Actaea racemosa* in a previous published study (Huang et al., 2010) but this has not yet been used to assess the effect of Sheng Ma extracts on liver cells. This assay did show a reduction in viability of the cells in all three extracts. The Sheng Ma "*Actaea cimicifuga"* extract reduced viability of the cells most of the three extracts across the three concentrations used. The *Actaea racemosa* extract reduced viability at the highest of the concentrations used and viability increased with decreased concentration. The Sheng Ma *"Actaea dahurica"* did not reduce viability as much as the other extracts. This was surprising as the chemical profile was similar to that of the other Sheng Ma extract.

The LDH assay was performed on both sets of cultured cells, HepG2 and HepaRG™ following treatment with the extracts. There were no significant changes in any of the treated HepaRG™ cells seen. The HepG2 cells however gave a very different result. The Sheng Ma extracts both caused the release of increased amounts of LDH particularly the Sheng Ma *"Actaea cimicifuga"* extract. This test indicates apoptosis and necrosis when LDH release is increased.

The three different cell culture assays give different clues about the toxicity of the extracts. The *Actaea racemosa* extract induced changes at the highest concentration with a reduction in viability of the cells seen in the MTT assay. There was no significant change in the LDH assay. The Sheng Ma extracts induced gene expression changes in line with several liver injury pathways. The LDH and MTT assays showed decreased viability of the cells and increased LDH release particularly when using

the HepG2 cell line. These results could show that another pathway other than those assessed with the hepatotoxicity array is involved.

The individual genes that were shown to be affected by the extracts could be explored further in the future. The cell culture assays as a whole showed that the Sheng Ma extracts had more of an adverse effect than that of the *Actaea racemosa* extract.

There are a number of key messages from this work. In spite of wide spread belief, it is possible to use DNA based testing for identification of plant species in commercially available herbal medicines. Following on from the loss of confidence in DNA testing due to the New York Attourney cease and desist of Black Cohosh products, this work has shown that it is possible to use DNA based testing for products even when made with alcohol extracts. This is contrary to the statements made by Parveen et al in their review of the use of barcoding for herbal products although they did discuss the potential of mini-barcode use (Parveen et al., 2016). This is further backed up with the fact that the British Pharmacopoeia is developing and investing in DNA based tests to incorporate into the herbal monographs. These developments will make it possible for the industry to utilise standardised, validated DNA methods that can be used with confidence and will complement the current chemistry/morphological based tests. The validated DNA assays will also help to overcome the existing issues that occur when using current methods. The DNA tests have shown that a number of commercially available Black Cohosh products are adulterated or substituted with Asian Cohosh species, and they identify species more accurately than chemical testing has shown. Finally ethanolic extracts of Asian Sheng Ma species show features consistent with them being more likely to be hepatotoxic that *A. racemosa*. There was previously not a study on this scale that aimed to attend to many of the questions and doubts surrounding Black Cohosh use. These major findings show that it is highly possible that adulterated products are the cause of liver injury rather than authentic products containing *A. racemosa.* The available literature suggests efficacy and safety from a clinical trial point of view when using high quality authentic Black Cohosh products. The conclusions of this thesis support the need for the UK to ensure that all Black Cohosh products available for use are of high

quality and are made only with *A. racemosa.* This is the surest route to ensure safety and confidence in the product.

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8 Appendix

8.1 GenBank sequence information

Table 66: A list of GenBank sequences for the nrITS region of *Actaea* species

Accession No.	Labelled species
GQ409511.1	*Actaea racemosa*
GQ409510.1	*Actaea racemosa*
GQ409509.1	*Actaea racemosa*
KU724194.1	*Actaea racemosa*
JQ033561.1	*Actaea racemosa*
JQ033560.1	*Actaea racemosa*
JQ033559.1	*Actaea racemosa*
JQ033558.1	*Actaea racemosa*
JQ033557.1	*Actaea racemosa*
JQ033556.1	*Actaea racemosa*
JQ033555.1	*Actaea racemosa*
JQ033554.1	*Actaea racemosa*
JQ033553.1	*Actaea racemosa*
JQ033552.1	*Actaea racemosa*
JQ033551.1	*Actaea racemosa*
JQ033550.1	*Actaea racemosa*
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JQ033546.1	*Actaea racemosa*
JQ033545.1	*Actaea racemosa*
JQ033544.1	*Actaea racemosa*
JQ033543.1	*Actaea racemosa*
JQ033542.1	*Actaea racemosa*
JQ033541.1	*Actaea racemosa*
JQ033540.1	*Actaea racemosa*
EU796898.1	*Actaea racemosa*
AB987687.1	*Actaea racemosa*
Z98296.1	*Actaea racemosa*
JQ033570.1	*Actaea rubra*
JQ033569.1	*Actaea rubra*
JQ033568.1	*Actaea rubra*
JQ033567.1	*Actaea rubra*
JQ033566.1	*Actaea rubra*
JQ033565.1	*Actaea rubra*
JQ033564.1	*Actaea rubra*
JQ033563.1	*Actaea rubra*
JQ033562.1	*Actaea rubra*
Z98278.1	*Actaea rubra*
KT598539.1	*Actaea dahurica*
KF233848.1	*Actaea dahurica*
KX674806.1	*Actaea dahurica*

KX674805.1	*Actaea dahurica*
KX674804.1	*Actaea dahurica*
KJ488053.1	*Actaea dahurica*
KJ488052.1	*Actaea dahurica*
KJ488051.1	*Actaea dahurica*
KJ488050.1	*Actaea dahurica*
KJ488049.1	*Actaea dahurica*
KJ488048.1	*Actaea dahurica*
KJ488047.1	*Actaea dahurica*
KJ488046.1	*Actaea dahurica*
KJ488045.1	*Actaea dahurica*
KJ488044.1	*Actaea dahurica*
KJ488043.1	*Actaea dahurica*
KJ488042.1	*Actaea dahurica*
KJ488041.1	*Actaea dahurica*
KJ488040.1	*Actaea dahurica*
KJ488039.1	*Actaea dahurica*
KJ488038.1	*Actaea dahurica*
KJ488037.1	*Actaea dahurica*
KJ488036.1	*Actaea dahurica*
KJ488035.1	*Actaea dahurica*
KJ488034.1	*Actaea dahurica*
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AB987685.1	*Actaea dahurica*
AB194179.1	*Actaea dahurica*
AB194178.1	*Actaea dahurica*
Z98284.1	*Actaea dahurica*
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KX675069.1	*Actaea heracleifolia*
KX675068.1	*Actaea heracleifolia*
KX675067.1	*Actaea heracleifolia*
KJ487985.1	*Actaea heracleifolia*
KJ487984.1	*Actaea heracleifolia*
KJ487983.1	*Actaea heracleifolia*

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KJ487981.1	*Actaea heracleifolia*
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KJ487976.1	*Actaea heracleifolia*
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KJ488009.1	*Actaea cimicifuga*
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KJ488007.1	*Actaea cimicifuga*
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KJ488005.1	*Actaea cimicifuga*
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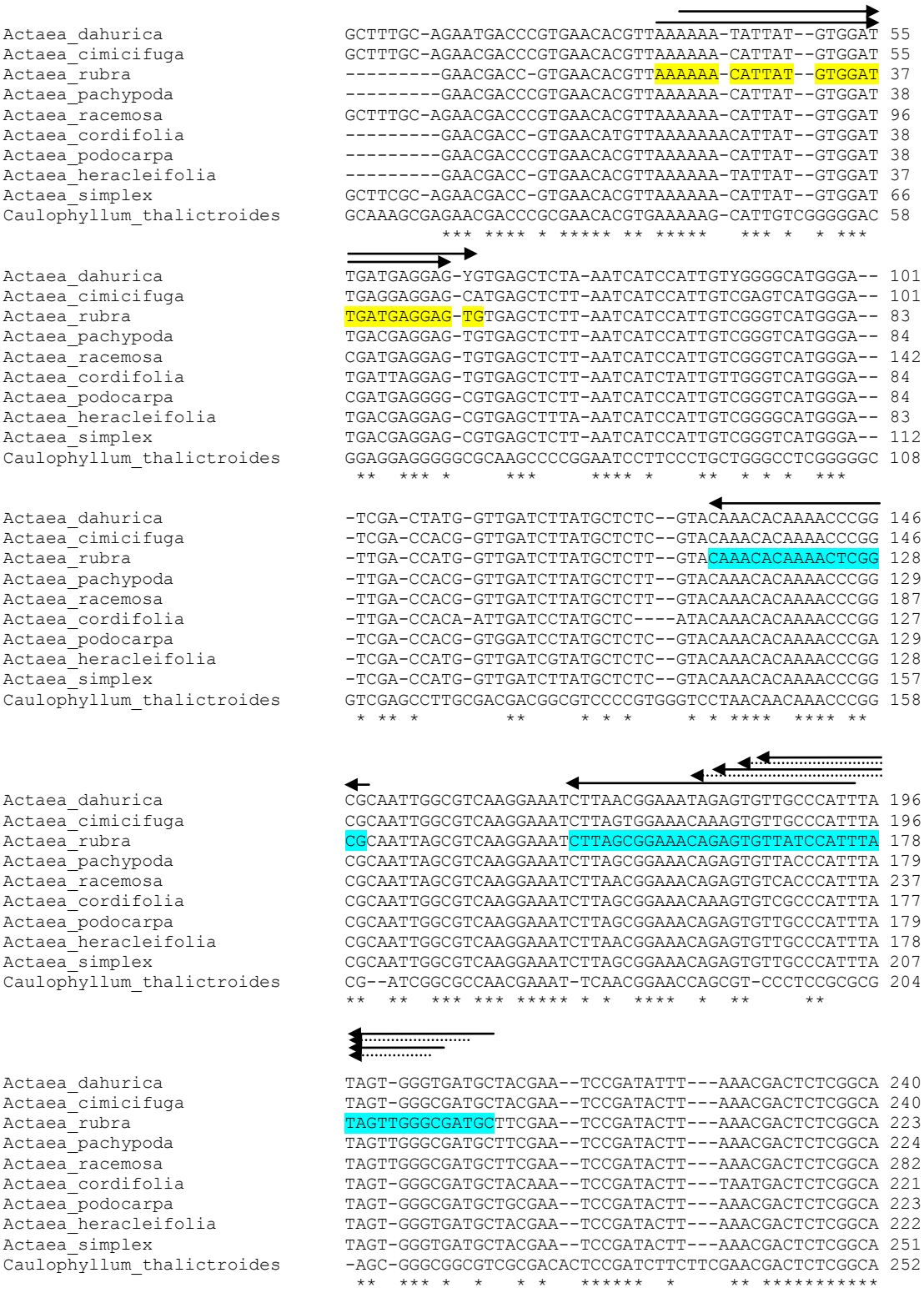
Z98298.1	*Actaea simplex*

8.2 Placement of species specific primers

8.2.1 Actaea rubra



8.2.2 Actaea pachypoda

```
Actaea_dahurica                GCTTTGC-AGAATGACCCGTGAACACGTTAAAAAA-TATTAT--GTGGAT 55
Actaea_cimicifuga              GCTTTGC-AGAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 55
Actaea_rubra                   ---------GAACGACC-GTGAACACGTTAAAAAA-CATTAT--GTGGAT 37
Actaea_pachypoda               ---------GAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 38
Actaea_racemosa                GCTTTGC-AGAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 96
Actaea_cordifolia              ---------GAACGACC-GTGAACATGTTAAAAAAACATTAT--GTGGAT 38
Actaea_podocarpa               ---------GAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 38
Actaea_heracleifolia           ---------GAACGACC-GTGAACACGTTAAAAAA-TATTAT--GTGGAT 37
Actaea_simplex                 GCTTCGC-AGAACGACC-GTGAACACGTTAAAAAA-CATTAT--GTGGAT 66
Caulophyllum_thalictroides     GCAAAGCGAGAACGACCCGCGAACACGTGAAAAAG-CATTGTCGGGGGAC 58
                                        *** **** * ***** ** *****   *** *  * ***
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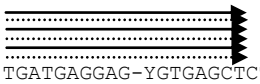

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Actaea_cimicifuga              TGAGGAGGAG-CATGAGCTCTT-AATCATCCATTGTCGAGTCATGGGA-- 101
Actaea_rubra                   TGATGAGGAG-TGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 83
Actaea_pachypoda               TGACGAGGAG-TGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 84
Actaea_racemosa                CGATGAGGAG-TGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 142
Actaea_cordifolia              TGATTAGGAG-TGTGAGCTCTT-AATCATCTATTGTTGGGTCATGGGA-- 84
Actaea_podocarpa               CGATGAGGGG-CGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 84
Actaea_heracleifolia           TGACGAGGAG-CGTGAGCTTTA-AATCATCCATTGTCGGGGCATGGGA-- 83
Actaea_simplex                 TGACGAGGAG-CGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 112
Caulophyllum_thalictroides     GGAGGAGGGGGCGCAAGCCCCGGAATCCTTCCCTGCTGGGCCTCGGGGGC 108
                                ** *** *     ***     **** *    **  * * *  ***

Actaea_dahurica                -TCGA-CTATG-GTTGATCTTATGCTCTC--GTACAAACACAAAACCCGG 146
Actaea_cimicifuga              -TCGA-CCACG-GTTGATCTTATGCTCTC--GTACAAACACAAAACCCGG 146
Actaea_rubra                   -TTGA-CCATG-GTTGATCTTATGCTCTT--GTACAAACACAAAACTCGG 128
Actaea_pachypoda               -TTGA-CCACG-GTTGATCTTATGCTCTT--GTACAAACACAAAACCCGG 129
Actaea_racemosa                -TTGA-CCACG-GTTGATCTTATGCTCTT--GTACAAACACAAAACCCGG 187
Actaea_cordifolia              -TTGA-CCACA-ATTGATCCTATGCTC----ATACAAACACAAAACCCGG 127
Actaea_podocarpa               -TCGA-CCACG-GTGGATCCTATGCTCTC--GTACAAACACAAAACCCGA 129
Actaea_heracleifolia           -TCGA-CCATG-GTTGATCGTATGCTCTC--GTACAAACACAAAACCCGG 128
Actaea_simplex                 -TCGA-CCATG-GTTGATCTTATGCTCTC--GTACAAACACAAAACCCGG 157
Caulophyllum_thalictroides     GTCGAGCCTTGCGACGACGGCGTCCCCGTGGGTCCTAACAACAAACCCGG 158
                                * ** *        **     * * *     * * ****  **** **
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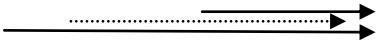

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Actaea_dahurica                CGCAATTGGCGTCAAGGAAATCTTAACGGAAATAGAGTGTTGCCCATTTA 196
Actaea_cimicifuga              CGCAATTGGCGTCAAGGAAATCTTAGTGGAAACAAAGTGTTGCCCATTTA 196
Actaea_rubra                   CGCAATTAGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTATCCATTTA 178
Actaea_pachypoda               CGCAATTAGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTACCCATTTA 179
Actaea_racemosa                CGCAATTAGCGTCAAGGAAATCTTAACGGAAACAGAGTGTCACCCATTTA 237
Actaea_cordifolia              CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAAAGTGTCGCCCATTTA 177
Actaea_podocarpa               CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTGCCCATTTA 179
Actaea_heracleifolia           CGCAATTGGCGTCAAGGAAATCTTAACGGAAACAGAGTGTTGCCCATTTA 178
Actaea_simplex                 CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTGCCCATTTA 207
Caulophyllum_thalictroides     CG--ATCGGCGCCAACGAAAT-TCAACGGAACCAGCGT-CCCTCCGCGCG 204
                               **  **  *** *** ***** * *  ****  *  **     **
```



```
Actaea_dahurica                TAGT-GGGTGATGCTACGAA--TCCGATATTT---AAACGACTCTCGGCA 240
Actaea_cimicifuga              TAGT-GGGCGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 240
Actaea_rubra                   TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 223
Actaea_pachypoda               TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 224
Actaea_racemosa                TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 282
Actaea_cordifolia              TAGT-GGGCGATGCTACAAA--TCCGATACTT---TAATGACTCTCGGCA 221
Actaea_podocarpa               TAGT-GGGCGATGCTGCGAA--TCCGATACTT---AAACGACTCTCGGCA 223
Actaea_heracleifolia           TAGT-GGGTGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 222
Actaea_simplex                 TAGT-GGGTGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 251
Caulophyllum_thalictroides     -AGC-GGGCGGCGTCGCGACACTCCGATCTTCTTCGAACGACTCTCGGCA 252
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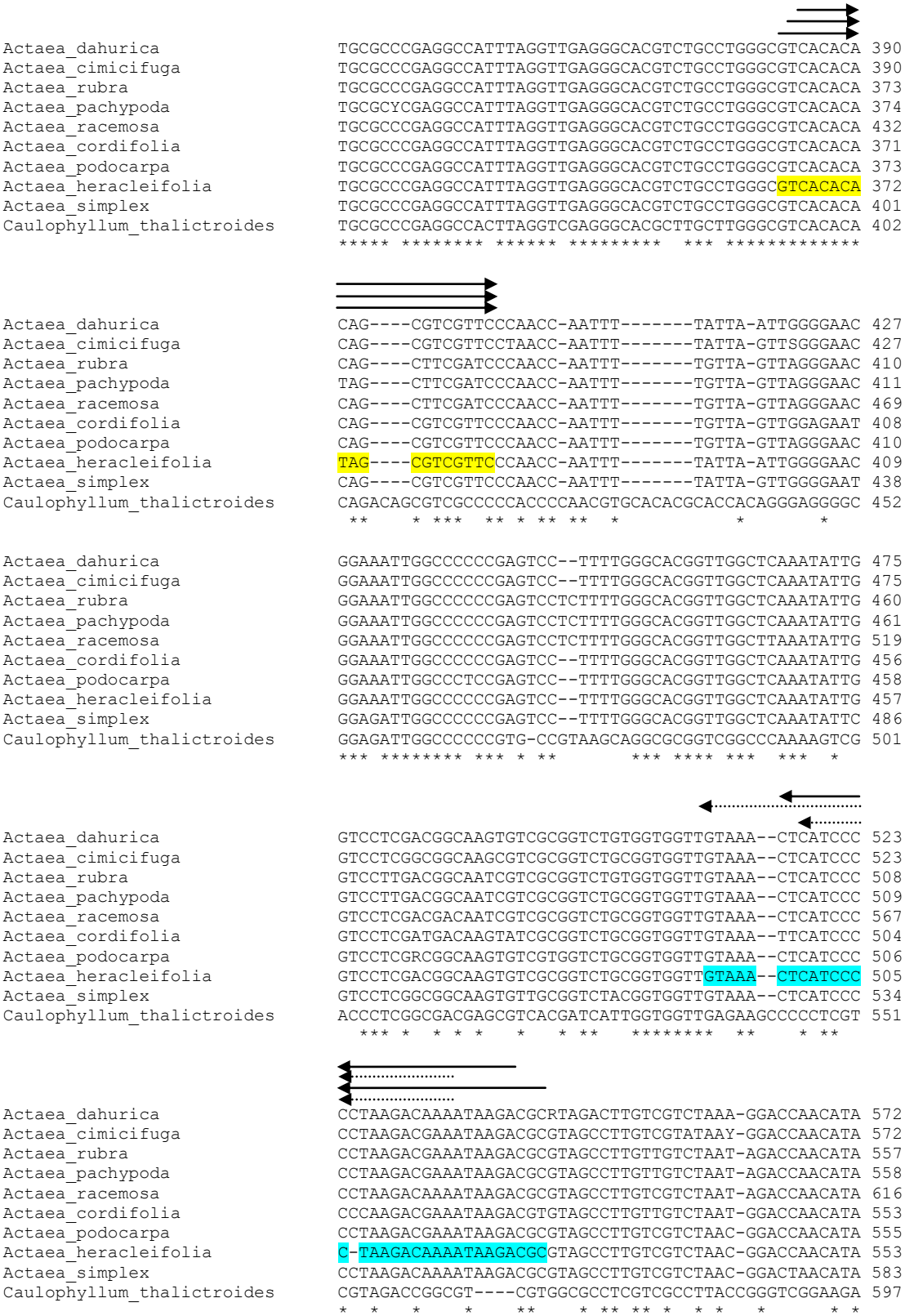
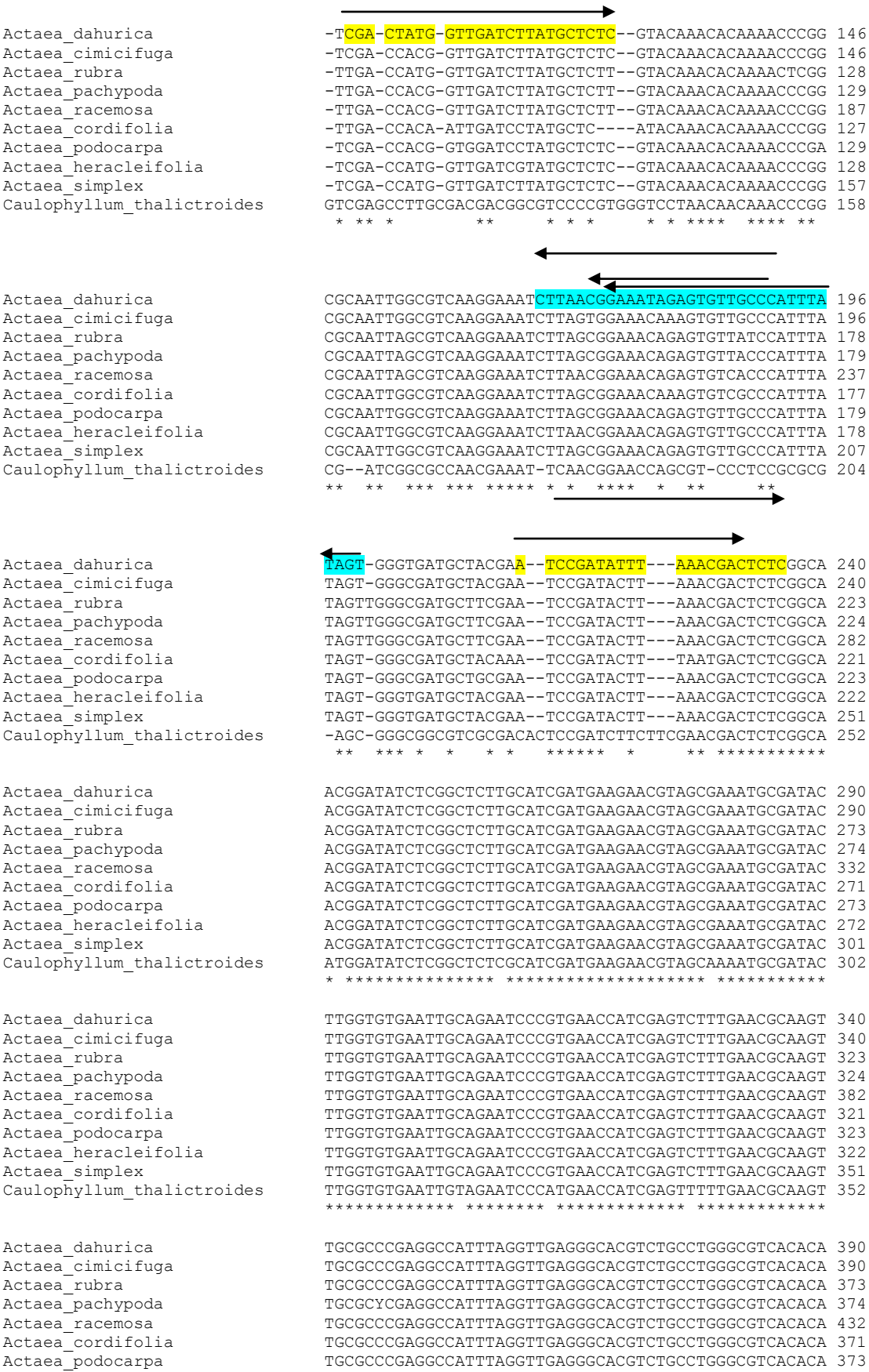
8.2.3 Actaea heracleifolia



8.2.4 Actaea dahurica



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Actaea_simplex                TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 401
Caulophyllum_thalictroides    TGCGCCCGAGGCCACTTAGGTCGAGGGCACGCTTGCTTGGGCGTCACACA 402
                              ***** ******** ****** *********  *** ************


Actaea_dahurica               CAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 427
Actaea_cimicifuga             CAG----CGTCGTTCCTAACC-AATTT-------TATTA-GTTSGGGAAC 427
Actaea_rubra                  CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_pachypoda              TAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 411
Actaea_racemosa               CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 469
Actaea_cordifolia             CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTGGAGAAT 408
Actaea_podocarpa              CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_heracleifolia          TAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 409
Actaea_simplex                CAG----CGTCGTTCCCAACC-AATTT-------TATTA-GTTGGGGAAT 438
Caulophyllum_thalictroides    CAGACAGCGTCGCCCCCACCCCAACGTGCACACGCACCACAGGGAGGGGC 452
                               **    * ***  ** * ** **  *           *       *


Actaea_dahurica               GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_cimicifuga             GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_rubra                  GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 460
Actaea_pachypoda              GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 461
Actaea_racemosa               GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTTAAATATTG 519
Actaea_cordifolia             GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 456
Actaea_podocarpa              GGAAATTGGCCCTCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 458
Actaea_heracleifolia          GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 457
Actaea_simplex                GGAGATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTC 486
Caulophyllum_thalictroides    GGAGATTGGCCCCCCGTG-CCGTAAGCAGGCGCGGTCGGCCCAAAAGTCG 501
                              *** ******** *** * **       *** **** ***  ***  *


Actaea_dahurica               GTCCTCGACGGCAAGTGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 523
Actaea_cimicifuga             GTCCTCGGCGGCAAGCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 523
Actaea_rubra                  GTCCTTGACGGCAATCGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 508
Actaea_pachypoda              GTCCTTGACGGCAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 509
Actaea_racemosa               GTCCTCGACGACAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 567
Actaea_cordifolia             GTCCTCGATGACAAGTATCGCGGTCTGCGGTGGTTGTAAA--TTCATCCC 504
Actaea_podocarpa              GTCCTCGRCGGCAAGTGTCGTGGTCTGCGGTGGTTGTAAA--CTCATCCC 506
Actaea_heracleifolia          GTCCTCGACGGCAAGTGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 505
Actaea_simplex                GTCCTCGGCGGCAAGTGTTGCGGTCTACGGTGGTTGTAAA--CTCATCCC 534
Caulophyllum_thalictroides    ACCCTCGGCGACGAGCGTCACGATCATTGGTGGTTGAGAAGCCCCCTCGT 551
                                *** *  * * *   *   * **   ********  **    * **


                                                                  <---------
                                                                <---------
Actaea_dahurica               CCTAAGACAAAATAAGACGCRTAGACTTGTCGTCTAAA-GGACCAACATA 572
Actaea_cimicifuga             CCTAAGACGAAATAAGACGCGTAGCCTTGTCGTATAAY-GGACCAACATA 572
Actaea_rubra                  CCTAAGACGAAATAAGACGCGTAGCCTTGTTGTCTAAT-AGACCAACATA 557
Actaea_pachypoda              CCTAAGACGAAATAAGACGCGTAGCCTTGTTGTCTAAT-AGACCAACATA 558
Actaea_racemosa               CCTAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAT-AGACCAACATA 616
Actaea_cordifolia             CCCAAGACGAAATAAGACGTGTAGCCTTGTTGTCTAAT-GGACCAACATA 553
Actaea_podocarpa              CCTAAGACGAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACCAACATA 555
Actaea_heracleifolia          C-TAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACCAACATA 553
Actaea_simplex                CCTAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACTAACATA 583
Caulophyllum_thalictroides    CGTAGACCGGCGT----CGTGGCGCCTCGTCGCCTTACCGGGTCGGAAGA 597
                              *  *   *    *    **    * ** ** *  * *   *      * *


                              <
                              <---
Actaea_dahurica               ACCCTTGGAAGCCGTTCAACGGTGTTCACCCT------------------- 604
Actaea_cimicifuga             ACCCTTRGAAGCCGTTCAACGGTTTTCACCCT------------------- 604
Actaea_rubra                  ACCCTTGGAAGCCGTTTAACGGTGTTCACCTTGCGACCCCAGGTCAGGCG 607
Actaea_pachypoda              ACCCTTGGAAGCCGTTTAACGGTGTTCACCTTGCGACCCCAGGTCAGGCG 608
Actaea_racemosa               ACCCTTGGAAGCCGTTTAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 666
Actaea_cordifolia             ACCCTTTGAAGCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 603
Actaea_podocarpa              CCCCTTATAATCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 605
Actaea_heracleifolia          ACCCTTGGAAGCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 603
Actaea_simplex                ACCCTTGGAAGCCGTTCATCGGTGTTCACCCTGCGACCCCAGGTCAGGCG 633
Caulophyllum_thalictroides    ACCCTC-----------------GTTTGTCCTGCA--------------- 615
                               ****                   **   * *
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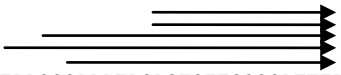
8.2.5 Actaea racemosa



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Actaea_dahurica              CGCAATTGGCGTCAAGGAAATCTTAACGGAAATAGAGTGTTGCCCATTTA 196
Actaea_cimicifuga            CGCAATTGGCGTCAAGGAAATCTTAGTGGAAACAAAGTGTTGCCCATTTA 196
Actaea_rubra                 CGCAATTAGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTATCCATTTA 178
Actaea_pachypoda             CGCAATTAGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTACCCATTTA 179
Actaea_racemosa              CGCAATTAGCGTCAAGGAAATCTTAACGGAAACAGAGTGTCACCCATTTA 237
Actaea_cordifolia            CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAAAGTGTCGCCCATTTA 177
Actaea_podocarpa             CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTGCCCATTTA 179
Actaea_heracleifolia         CGCAATTGGCGTCAAGGAAATCTTAACGGAAACAGAGTGTTGCCCATTTA 178
Actaea_simplex               CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTGCCCATTTA 207
Caulophyllum_thalictroides   CG--ATCGGCGCCAACGAAAT-TCAACGGAACCAGCGT-CCCTCCGCGCG 204
                             **  **  *** *** ***** * *  ****  *  **     **

Actaea_dahurica              TAGT-GGGTGATGCTACGAA--TCCGATATTT---AAACGACTCTCGGCA 240
Actaea_cimicifuga            TAGT-GGGCGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 240
Actaea_rubra                 TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 223
Actaea_pachypoda             TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 224
Actaea_racemosa              TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 282
Actaea_cordifolia            TAGT-GGGCGATGCTACAAA--TCCGATACTT---TAATGACTCTCGGCA 221
Actaea_podocarpa             TAGT-GGGCGATGCTGCGAA--TCCGATACTT---AAACGACTCTCGGCA 223
Actaea_heracleifolia         TAGT-GGGTGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 222
Actaea_simplex               TAGT-GGGTGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 251
Caulophyllum_thalictroides   -AGC-GGGCGGCGTCGCGACACTCCGATCTTCTTCGAACGACTCTCGGCA 252
                              **  *** *  *   * *   ******  *     ** ***********

Actaea_dahurica              ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 290
Actaea_cimicifuga            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 290
Actaea_rubra                 ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_pachypoda             ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 274
Actaea_racemosa              ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 332
Actaea_cordifolia            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 271
Actaea_podocarpa             ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_heracleifolia         ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 272
Actaea_simplex               ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 301
Caulophyllum_thalictroides   ATGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATAC 302
                             * *************** ******************** ***********

Actaea_dahurica              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_cimicifuga            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_rubra                 TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_pachypoda             TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 324
Actaea_racemosa              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 382
Actaea_cordifolia            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 321
Actaea_podocarpa             TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_heracleifolia         TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 322
Actaea_simplex               TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 351
Caulophyllum_thalictroides   TTGGTGTGAATTGTAGAATCCCATGAACCATCGAGTTTTTGAACGCAAGT 352
                             ************* ******** ************* *************

Actaea_dahurica              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_cimicifuga            TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_rubra                 TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_pachypoda             TGCGCYCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 374
Actaea_racemosa              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 432
Actaea_cordifolia            TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 371
Actaea_podocarpa             TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_heracleifolia         TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 372
Actaea_simplex               TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 401
Caulophyllum_thalictroides   TGCGCCCGAGGCCACTTAGGTCGAGGGCACGCTTGCTTGGGCGTCACACA 402
                             ***** ******** ****** *********  *** *************

Actaea_dahurica              CAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 427
Actaea_cimicifuga            CAG----CGTCGTTCCTAACC-AATTT-------TATTA-GTTSGGGAAC 427
Actaea_rubra                 CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_pachypoda             TAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 411
Actaea_racemosa              CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 469
Actaea_cordifolia            CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTGGAGAAT 408
Actaea_podocarpa             CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
```

```
Actaea_heracleifolia            TAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 409
Actaea_simplex                  CAG----CGTCGTTCCCAACC-AATTT-------TATTA-GTTGGGGAAT 438
Caulophyllum_thalictroides      CAGACAGCGTCGCCCCCACCCCAACGTGCACACGCACCACAGGGAGGGGC 452
                                 **    * ***  ** * ** **  *           *      *

                                                                    <--------
                                                                    <.......
                                                                    <--------
                                                  <.................
Actaea_dahurica                 GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_cimicifuga               GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_rubra                    GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 460
Actaea_pachypoda                GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 461
Actaea_racemosa                 GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTTAAATATTG 519
Actaea_cordifolia               GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 456
Actaea_podocarpa                GGAAATTGGCCCTCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 458
Actaea_heracleifolia            GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 457
Actaea_simplex                  GGAGATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTC 486
Caulophyllum_thalictroides      GGAGATTGGCCCCCCCGTG-CCGTAAGCAGGCGCGGTCGGCCCAAAAGTCG 501
                                *** ******** *** * **       *** **** ***  ***  *

                                <--------
                                <.......
                                <--------
Actaea_dahurica                 GTCCTCGACGGCAAGTGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 523
Actaea_cimicifuga               GTCCTCGGCGGCAAGCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 523
Actaea_rubra                    GTCCTTGACGGCAATCGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 508
Actaea_pachypoda                GTCCTTGACGGCAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 509
Actaea_racemosa                 GTCCTCGACGACAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 567
Actaea_cordifolia               GTCCTCGATGACAAGTATCGCGGTCTGCGGTGGTTGTAAA--TTCATCCC 504
Actaea_podocarpa                GTCCTCGRCGGCAAGTGTCGTGGTCTGCGGTGGTTGTAAA--CTCATCCC 506
Actaea_heracleifolia            GTCCTCGACGGCAAGTGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 505
Actaea_simplex                  GTCCTCGGCGGCAAGTGTTGCGGTCTACGGTGGTTGTAAA--CTCATCCC 534
Caulophyllum_thalictroides      ACCCTCGGCGACGAGCGTCACGATCATTGGTGGTTGAGAAGCCCCCTCGT 551
                                  *** *  * * *   *   * **   ********  **    * **
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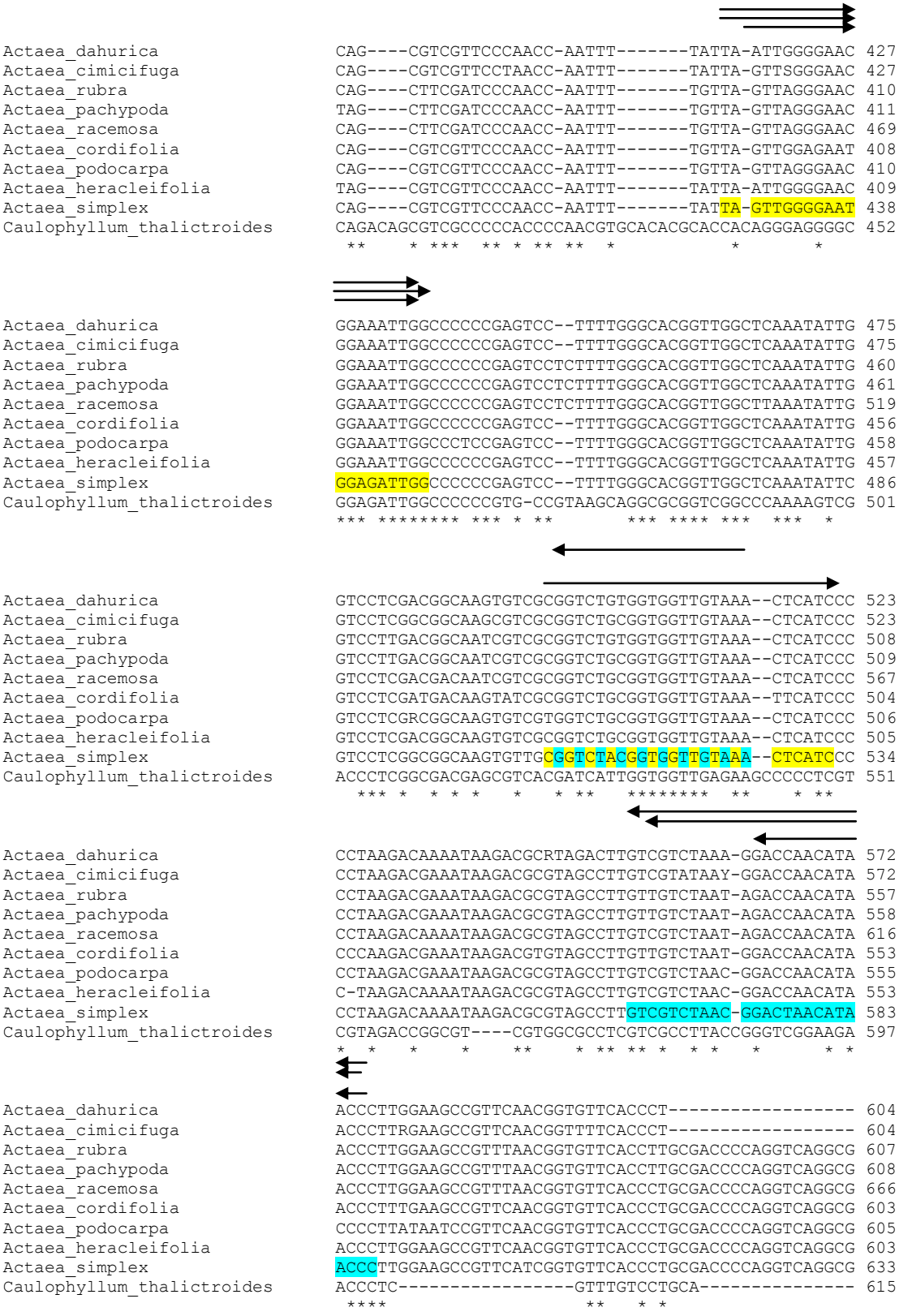
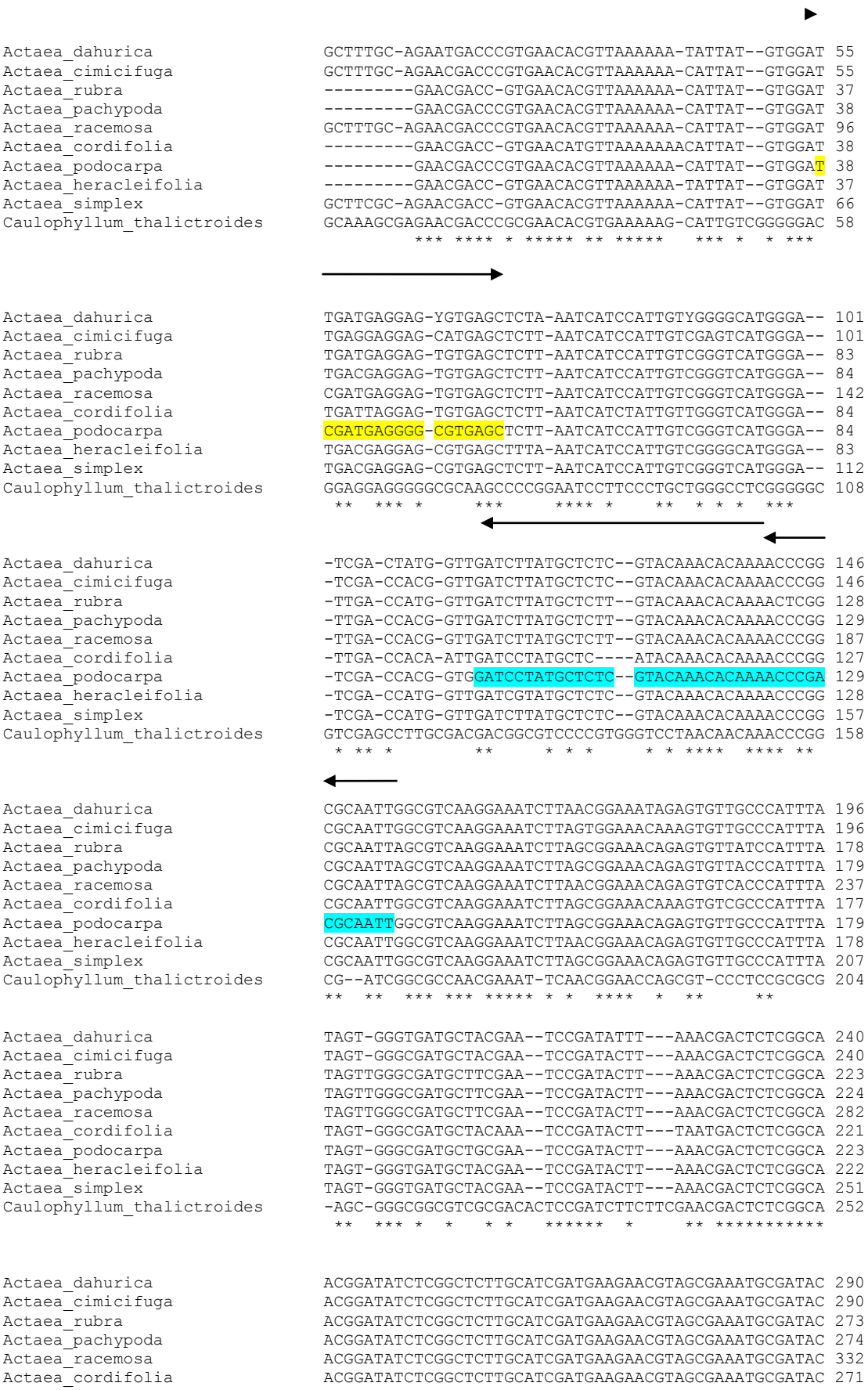
8.2.6 *Actaea simplex*



8.2.7 Actaea podocarpa



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Actaea_heracleifolia            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 272
Actaea_simplex                  ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 301
Caulophyllum_thalictroides      ATGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATAC 302
                                * *************** ******************* ***********

Actaea_dahurica                 TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_cimicifuga               TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_rubra                    TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_pachypoda                TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 324
Actaea_racemosa                 TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 382
Actaea_cordifolia               TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 321
Actaea_podocarpa                TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_heracleifolia            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 322
Actaea_simplex                  TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 351
Caulophyllum_thalictroides      TTGGTGTGAATTGTAGAATCCCATGAACCATCGAGTTTTTGAACGCAAGT 352
                                ************* ******** ************* *************

Actaea_dahurica                 TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_cimicifuga               TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_rubra                    TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_pachypoda                TGCGCYCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 374
Actaea_racemosa                 TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 432
Actaea_cordifolia               TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 371
Actaea_podocarpa                TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_heracleifolia            TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 372
Actaea_simplex                  TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 401
Caulophyllum_thalictroides      TGCGCCCGAGGCCACTTAGGTCGAGGGCACGCTTGCTTGGGCGTCACACA 402
                                ***** ******** ****** *********  *** *************

Actaea_dahurica                 CAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 427
Actaea_cimicifuga               CAG----CGTCGTTCCTAACC-AATTT-------TATTA-GTTSGGGAAC 427
Actaea_rubra                    CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_pachypoda                TAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 411
Actaea_racemosa                 CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 469
Actaea_cordifolia               CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTGGAGAAT 408
Actaea_podocarpa                CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_heracleifolia            TAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 409
Actaea_simplex                  CAG----CGTCGTTCCCAACC-AATTT-------TATTA-GTTGGGGAAT 438
Caulophyllum_thalictroides      CAGACAGCGTCGCCCCCACCCCAACGTGCACACGCACCACAGGGAGGGGC 452
                                 **    * ***  ** * ** **  *           *       *

Actaea_dahurica                 GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_cimicifuga               GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_rubra                    GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 460
Actaea_pachypoda                GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 461
Actaea_racemosa                 GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTTAAATATTG 519
Actaea_cordifolia               GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 456
Actaea_podocarpa                GGAAATTGGCCCTCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 458
Actaea_heracleifolia            GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 457
Actaea_simplex                  GGAGATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTC 486
Caulophyllum_thalictroides      GGAGATTGGCCCCCCGTG-CCGTAAGCAGGCGCGGTCGGCCCAAAAGTCG 501
                                *** ******** *** * **       *** **** ***  ***  *

                                          ---------->
Actaea_dahurica                 GTCCTCGACGGCAAGTGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 523
Actaea_cimicifuga               GTCCTCGGCGGCAAGCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 523
Actaea_rubra                    GTCCTTGACGGCAATCGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 508
Actaea_pachypoda                GTCCTTGACGGCAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 509
Actaea_racemosa                 GTCCTCGACGACAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 567
Actaea_cordifolia               GTCCTCGATGACAAGTATCGCGGTCTGCGGTGGTTGTAAA--TTCATCCC 504
Actaea_podocarpa                GTCCTCGRCGGCAAGTGTCGTGGTCTGCGGTGGTTGTAAA--CTCATCCC 506
Actaea_heracleifolia            GTCCTCGACGGCAAGTGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 505
Actaea_simplex                  GTCCTCGGCGGCAAGTGTTGCGGTCTACGGTGGTTGTAAA--CTCATCCC 534
Caulophyllum_thalictroides      ACCCTCGGCGACGAGCGTCACGATCATTGGTGGTTGAGAAGCCCCCTCGT 551
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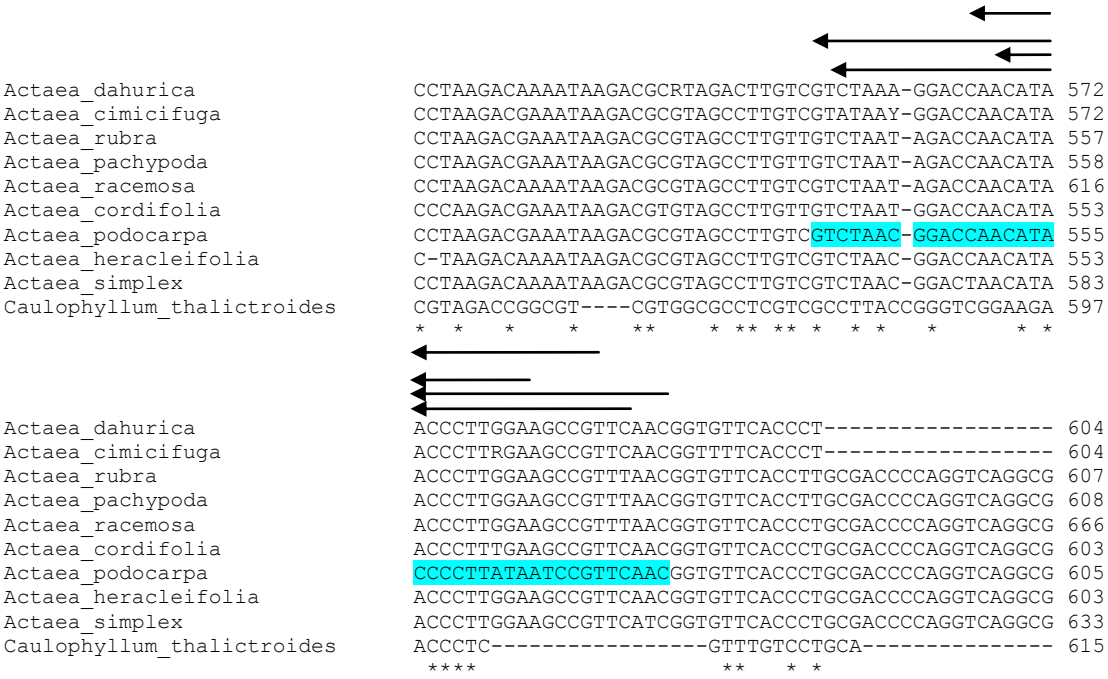
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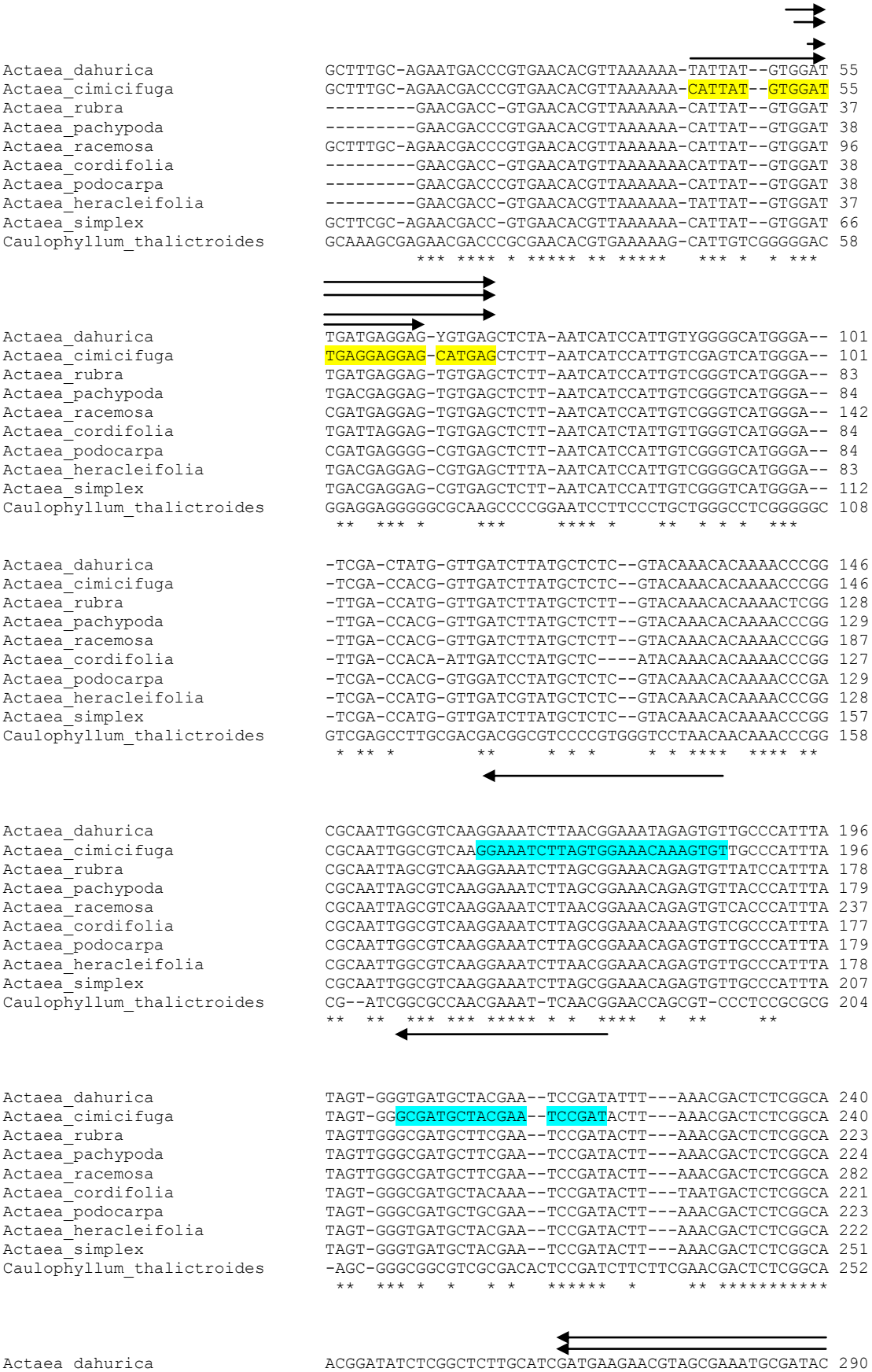
Actaea_cimicifuga CCTAAGACGAAATAAGACGCGTAGCCTTGTCGTATAAY-GGACCAACATA 572
Actaea_rubra CCTAAGACGAAATAAGACGCGTAGCCTTGTTGTCTAAT-AGACCAACATA 557
Actaea_pachypoda CCTAAGACGAAATAAGACGCGTAGCCTTGTTGTCTAAT-AGACCAACATA 558
Actaea_racemosa CCTAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAT-AGACCAACATA 616
Actaea_cordifolia CCCAAGACGAAATAAGACGTGTAGCCTTGTTGTCTAAT-GGACCAACATA 553
Actaea_podocarpa CCTAAGACGAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACCAACATA 555
Actaea_heracleifolia C-TAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACCAACATA 553
Actaea_simplex CCTAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACTAACATA 583
Caulophyllum_thalictroides CGTAGACCGGCGT----CGTGGCGCCTCGTCGCCTTACCGGGTCGGAAGA 597
Actaea_dahurica ACCCTTGGAAGCCGTTCAACGGTGTTCACCCT------------------ 604
Actaea_cimicifuga ACCCTTRGAAGCCGTTCAACGGTTTTCACCCT------------------ 604
Actaea_rubra ACCCTTGGAAGCCGTTTAACGGTGTTCACCTTGCGACCCCAGGTCAGGCG 607
Actaea_pachypoda ACCCTTGGAAGCCGTTTAACGGTGTTCACCTTGCGACCCCAGGTCAGGCG 608
Actaea_racemosa ACCCTTGGAAGCCGTTTAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 666
Actaea_cordifolia ACCCTTTGAAGCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 603
Actaea_podocarpa CCCCTTATAATCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 605
Actaea_heracleifolia ACCCTTGGAAGCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 603
Actaea_simplex ACCCTTGGAAGCCGTTCATCGGTGTTCACCCTGCGACCCCAGGTCAGGCG 633
Caulophyllum_thalictroides ACCCTC-----------------GTTTGTCCTGCA---------------- 615

8.2.8 Actaea cimicifuga



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Actaea_pachypoda            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 274
Actaea_racemosa             ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 332
Actaea_cordifolia           ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 271
Actaea_podocarpa            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_heracleifolia        ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 272
Actaea_simplex              ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 301
Caulophyllum_thalictroides  ATGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATAC 302
                            * *************** ******************** ***********

                                                ◄
Actaea_dahurica             TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_cimicifuga           TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_rubra                TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_pachypoda            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 324
Actaea_racemosa             TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 382
Actaea_cordifolia           TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 321
Actaea_podocarpa            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_heracleifolia        TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 322
Actaea_simplex              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 351
Caulophyllum_thalictroides  TTGGTGTGAATTGTAGAATCCCATGAACCATCGAGTTTTTGAACGCAAGT 352
                            ************* ******** ************* *************

                                                                  ──►
Actaea_dahurica             TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_cimicifuga           TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_rubra                TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_pachypoda            TGCGCYCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 374
Actaea_racemosa             TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 432
Actaea_cordifolia           TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 371
Actaea_podocarpa            TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_heracleifolia        TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 372
Actaea_simplex              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 401
Caulophyllum_thalictroides  TGCGCCCGAGGCCACTTAGGTCGAGGGCACGCTTGCTTGGGCGTCACACA 402
                            ***** ******** ****** *********  *** *************

                                  ──────────────────►
Actaea_dahurica             CAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 427
Actaea_cimicifuga           CAG----CGTCGTTCCTAACC-AATTT-------TATTA-GTTSGGGAAC 427
Actaea_rubra                CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_pachypoda            TAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 411
Actaea_racemosa             CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 469
Actaea_cordifolia           CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTGGAGAAT 408
Actaea_podocarpa            CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_heracleifolia        TAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 409
Actaea_simplex              CAG----CGTCGTTCCCAACC-AATTT-------TATTA-GTTGGGGAAT 438
Caulophyllum_thalictroides  CAGACAGCGTCGCCCCCACCCCAACGTGCACACGCACCACAGGGAGGGGC 452
                             **    * ***  ** * ** **  *           *       *

Actaea_dahurica             GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_cimicifuga           GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_rubra                GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 460
Actaea_pachypoda            GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 461
Actaea_racemosa             GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTTAAATATTG 519
Actaea_cordifolia           GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 456
Actaea_podocarpa            GGAAATTGGCCCTCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 458
Actaea_heracleifolia        GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 457
Actaea_simplex              GGAGATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTC 486
Caulophyllum_thalictroides  GGAGATTGGCCCCCCGTG-CCGTAAGCAGGCGCGGTCGGCCCAAAAGTCG 501
                            *** ******** *** * **       *** **** ***  ***  *
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Actaea_dahurica                GTCCTCGACGGCAAGTGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 523
Actaea_cimicifuga              GTCCTCGGCGGCAAGCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 523
Actaea_rubra                   GTCCTTGACGGCAATCGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 508
Actaea_pachypoda               GTCCTTGACGGCAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 509
Actaea_racemosa                GTCCTCGACGACAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 567
Actaea_cordifolia              GTCCTCGATGACAAGTATCGCGGTCTGCGGTGGTTGTAAA--TTCATCCC 504
Actaea_podocarpa               GTCCTCGRCGGCAAGTGTCGTGGTCTGCGGTGGTTGTAAA--CTCATCCC 506
Actaea_heracleifolia           GTCCTCGACGGCAAGTGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 505
Actaea_simplex                 GTCCTCGGCGGCAAGTGTTGCGGTCTACGGTGGTTGTAAA--CTCATCCC 534
Caulophyllum_thalictroides     ACCCTCGGCGACGAGCGTCACGATCATTGGTGGTTGAGAAGCCCCCTCGT 551
                                 *** *  * * *   *   * **   ********  **    * **
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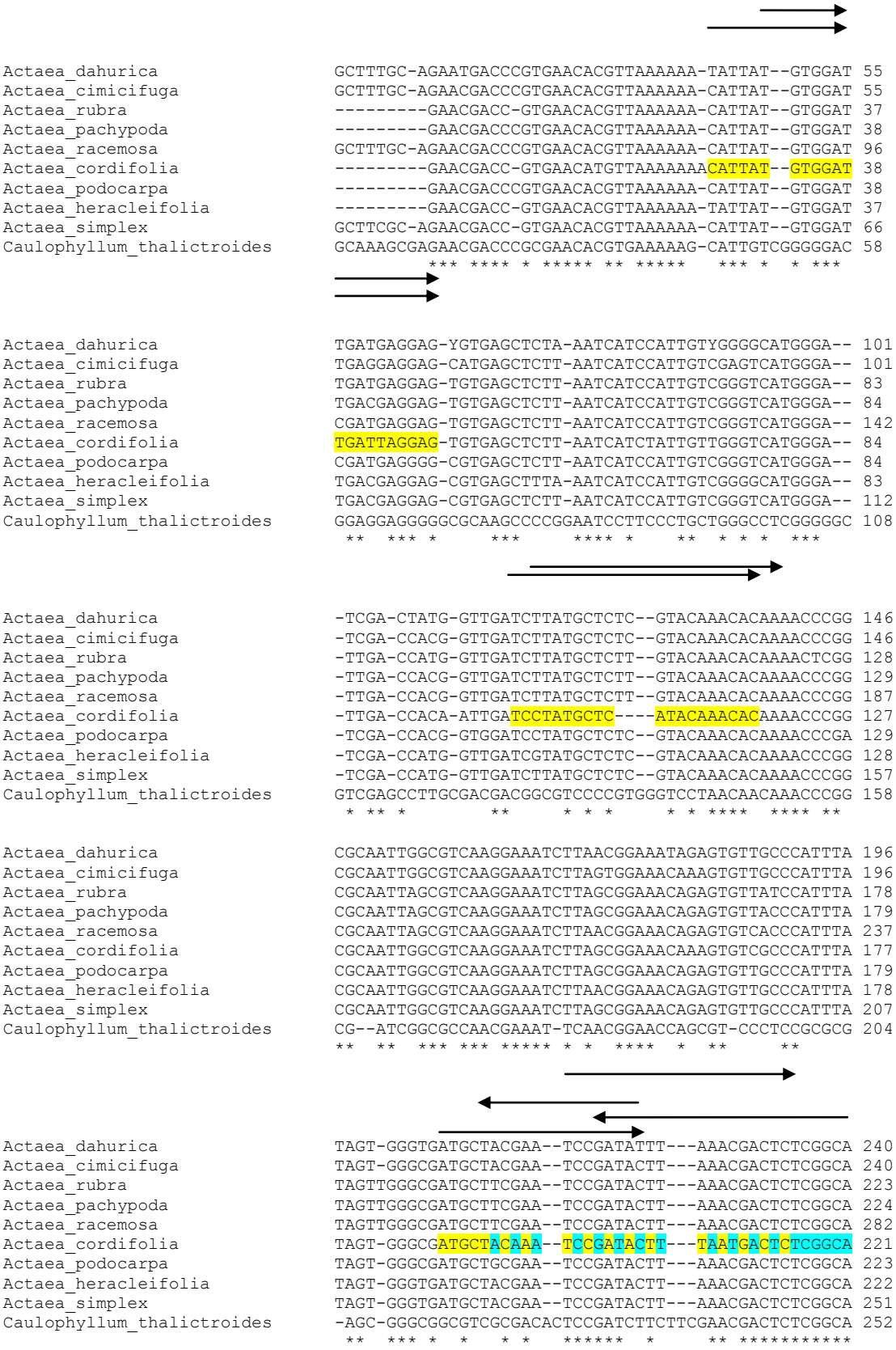
8.2.9 *Actaea cordifolia*



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Actaea_cimicifuga            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 290
Actaea_rubra                 ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_pachypoda             ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 274
Actaea_racemosa              ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 332
Actaea_cordifolia            ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 271
Actaea_podocarpa             ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_heracleifolia         ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 272
Actaea_simplex               ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 301
Caulophyllum_thalictroides   ATGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATAC 302
                             * *************** ******************** ***********

Actaea_dahurica              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_cimicifuga            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_rubra                 TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_pachypoda             TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 324
Actaea_racemosa              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 382
Actaea_cordifolia            TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 321
Actaea_podocarpa             TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_heracleifolia         TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 322
Actaea_simplex               TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 351
Caulophyllum_thalictroides   TTGGTGTGAATTGTAGAATCCCATGAACCATCGAGTTTTTGAACGCAAGT 352
                             ************* ******** ************* *************

Actaea_dahurica              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_cimicifuga            TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_rubra                 TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_pachypoda             TGCGCYCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 374
Actaea_racemosa              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 432
Actaea_cordifolia            TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 371
Actaea_podocarpa             TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_heracleifolia         TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 372
Actaea_simplex               TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 401
Caulophyllum_thalictroides   TGCGCCCGAGGCCACTTAGGTCGAGGGCACGCTTGCTTGGGCGTCACACA 402
                             ***** ******** ****** *********  *** *************

                                                                  ──────────►
Actaea_dahurica              CAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 427
Actaea_cimicifuga            CAG----CGTCGTTCCTAACC-AATTT-------TATTA-GTTSGGGAAC 427
Actaea_rubra                 CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_pachypoda             TAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 411
Actaea_racemosa              CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 469
Actaea_cordifolia            CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTGGAGAAT 408
Actaea_podocarpa             CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_heracleifolia         TAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 409
Actaea_simplex               CAG----CGTCGTTCCCAACC-AATTT-------TATTA-GTTGGGGAAT 438
Caulophyllum_thalictroides   CAGACAGCGTCGCCCCCACCCCAACGTGCACACGCACCACAGGGAGGGGC 452
                              **    * ***  ** * ** **  *           *       *

                                                  ──►
Actaea_dahurica              GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_cimicifuga            GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_rubra                 GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 460
Actaea_pachypoda             GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 461
Actaea_racemosa              GGAAATTGGCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTTAAATATTG 519
Actaea_cordifolia            GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 456
Actaea_podocarpa             GGAAATTGGCCCTCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 458
Actaea_heracleifolia         GGAAATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 457
Actaea_simplex               GGAGATTGGCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTC 486
Caulophyllum_thalictroides   GGAGATTGGCCCCCCGTG-CCGTAAGCAGGCGCGGTCGGCCCAAAAGTCG 501
                             *** ******** *** * **       *** **** ***  ***  *
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8.2.10 Caulophyllum thalictroides

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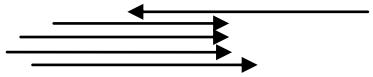
Actaea_dahurica                GCTTTGC-AGAATGACCCGTGAACACGTTAAAAAA-TATTAT--GTGGAT 55
Actaea_cimicifuga              GCTTTGC-AGAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 55
Actaea_rubra                   ---------GAACGACC-GTGAACACGTTAAAAAA-CATTAT--GTGGAT 37
Actaea_pachypoda               ---------GAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 38
Actaea_racemosa                GCTTTGC-AGAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 96
Actaea_cordifolia              ---------GAACGACC-GTGAACATGTTAAAAAAACATTAT--GTGGAT 38
Actaea_podocarpa               ---------GAACGACCCGTGAACACGTTAAAAAA-CATTAT--GTGGAT 38
Actaea_heracleifolia           ---------GAACGACC-GTGAACACGTTAAAAAA-TATTAT--GTGGAT 37
Actaea_simplex                 GCTTCGC-AGAACGACC-GTGAACACGTTAAAAAA-CATTAT--GTGGAT 66
Caulophyllum_thalictroides     GCAAAGCGAGAACGACCCGCGAACACGTGAAAAAG-CATTGTCGGGGGAC 58
                                         *** **** * ***** ** *****   *** *  * ***

Actaea_dahurica                TGATGAGGAG-YGTGAGCTCTA-AATCATCCATTGTYGGGGCATGGGA-- 101
Actaea_cimicifuga              TGAGGAGGAG-CATGAGCTCTT-AATCATCCATTGTCGAGTCATGGGA-- 101
Actaea_rubra                   TGATGAGGAG-TGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 83
Actaea_pachypoda               TGACGAGGAG-TGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 84
Actaea_racemosa                CGATGAGGAG-TGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 142
Actaea_cordifolia              TGATTAGGAG-TGTGAGCTCTT-AATCATCTATTGTTGGGTCATGGGA-- 84
Actaea_podocarpa               CGATGAGGGG-CGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 84
Actaea_heracleifolia           TGACGAGGAG-CGTGAGCTTTA-AATCATCCATTGTCGGGGCATGGGA-- 83
Actaea_simplex                 TGACGAGGAG-CGTGAGCTCTT-AATCATCCATTGTCGGGTCATGGGA-- 112
Caulophyllum_thalictroides     GGAGGAGGGGGCGCAAGCCCCGGAATCCTTCCCTGCTGGGCCTCGGGGGC 108
                              **  *** *     ***     **** *    **  * * *  ***

Actaea_dahurica                -TCGA-CTATG-GTTGATCTTATGCTCTC--GTACAAACACAAAACCCGG 146
Actaea_cimicifuga              -TCGA-CCACG-GTTGATCTTATGCTCTC--GTACAAACACAAAACCCGG 146
Actaea_rubra                   -TTGA-CCATG-GTTGATCTTATGCTCTT--GTACAAACACAAAACTCGG 128
Actaea_pachypoda               -TTGA-CCACG-GTTGATCTTATGCTCTT--GTACAAACACAAAACCCGG 129
Actaea_racemosa                -TTGA-CCACG-GTTGATCTTATGCTCTT--GTACAAACACAAAACCCGG 187
Actaea_cordifolia              -TTGA-CCACA-ATTGATCCTATGCTC----ATACAAACACAAAACCCGG 127
Actaea_podocarpa               -TCGA-CCACG-GTGGATCCTATGCTCTC--GTACAAACACAAAACCCGA 129
Actaea_heracleifolia           -TCGA-CCATG-GTTGATCGTATGCTCTC--GTACAAACACAAAACCCGG 128
Actaea_simplex                 -TCGA-CCATG-GTTGATCTTATGCTCTC--GTACAAACACAAAACCCGG 157
Caulophyllum_thalictroides     GTCGAGCCTTGCGACGACGGCGTCCCCGTGGGTCCTAACAACAAACCCGG 158
                                * ** *        **     * * *     * * ****  **** **

Actaea_dahurica                CGCAATTGGCGTCAAGGAAATCTTAACGGAAATAGAGTGTTGCCCATTTA 196
Actaea_cimicifuga              CGCAATTGGCGTCAAGGAAATCTTAGTGGAAACAAAGTGTTGCCCATTTA 196
Actaea_rubra                   CGCAATTAGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTATCCATTTA 178
Actaea_pachypoda               CGCAATTAGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTACCCATTTA 179
Actaea_racemosa                CGCAATTAGCGTCAAGGAAATCTTAACGGAAACAGAGTGTCACCCATTTA 237
Actaea_cordifolia              CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAAAGTGTCGCCCATTTA 177
Actaea_podocarpa               CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTGCCCATTTA 179
Actaea_heracleifolia           CGCAATTGGCGTCAAGGAAATCTTAACGGAAACAGAGTGTTGCCCATTTA 178
Actaea_simplex                 CGCAATTGGCGTCAAGGAAATCTTAGCGGAAACAGAGTGTTGCCCATTTA 207
Caulophyllum_thalictroides     CG--ATCGGCGCCAACGAAAT-TCAACGGAACCAGCGT-CCCTCCGCGCG 204
                               **  **  *** *** ***** * *  ****  *  **     **

Actaea_dahurica                TAGT-GGGTGATGCTACGAA--TCCGATATTT---AAACGACTCTCGGCA 240
Actaea_cimicifuga              TAGT-GGGCGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 240
Actaea_rubra                   TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 223
Actaea_pachypoda               TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 224
Actaea_racemosa                TAGTTGGGCGATGCTTCGAA--TCCGATACTT---AAACGACTCTCGGCA 282
Actaea_cordifolia              TAGT-GGGCGATGCTACAAA--TCCGATACTT---TAATGACTCTCGGCA 221
Actaea_podocarpa               TAGT-GGGCGATGCTGCGAA--TCCGATACTT---AAACGACTCTCGGCA 223
Actaea_heracleifolia           TAGT-GGGTGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 222
Actaea_simplex                 TAGT-GGGTGATGCTACGAA--TCCGATACTT---AAACGACTCTCGGCA 251
Caulophyllum_thalictroides     -AGC-GGGCGGCGTCGCGACACTCCGATCTTCTTCGAACGACTCTCGGCA 252
                                **  *** *  *   * *   ******  *     ** ***********


Actaea_dahurica                ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 290
Actaea_cimicifuga              ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 290
Actaea_rubra                   ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_pachypoda               ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 274
Actaea_racemosa                ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 332
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Actaea_cordifolia              ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 271
Actaea_podocarpa               ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 273
Actaea_heracleifolia           ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 272
Actaea_simplex                 ACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATAC 301
Caulophyllum_thalictroides     ATGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATAC 302
                               * *************** ******************** ***********

Actaea_dahurica                TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_cimicifuga              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 340
Actaea_rubra                   TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_pachypoda               TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 324
Actaea_racemosa                TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 382
Actaea_cordifolia              TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 321
Actaea_podocarpa               TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 323
Actaea_heracleifolia           TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 322
Actaea_simplex                 TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGT 351
Caulophyllum_thalictroides     TTGGTGTGAATTGTAGAATCCCATGAACCATCGAGTTTTTGAACGCAAGT 352
                               ************* ******** ************* *************

Actaea_dahurica                TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_cimicifuga              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 390
Actaea_rubra                   TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_pachypoda               TGCGCYCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 374
Actaea_racemosa                TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 432
Actaea_cordifolia              TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 371
Actaea_podocarpa               TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 373
Actaea_heracleifolia           TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 372
Actaea_simplex                 TGCGCCCGAGGCCATTTAGGTTGAGGGCACGTCTGCCTGGGCGTCACACA 401
Caulophyllum_thalictroides     TGCGCCCGAGGCCACTTAGGTCGAGGGCACGCTTGCTTGGGCGTCACACA 402
                               ***** ******** ****** *********  *** **************

Actaea_dahurica                CAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 427
Actaea_cimicifuga              CAG----CGTCGTTCCTAACC-AATTT-------TATTA-GTTSGGGAAC 427
Actaea_rubra                   CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_pachypoda               TAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 411
Actaea_racemosa                CAG----CTTCGATCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 469
Actaea_cordifolia              CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTGGAGAAT 408
Actaea_podocarpa               CAG----CGTCGTTCCCAACC-AATTT-------TGTTA-GTTAGGGAAC 410
Actaea_heracleifolia           TAG----CGTCGTTCCCAACC-AATTT-------TATTA-ATTGGGGAAC 409
Actaea_simplex                 CAG----CGTCGTTCCCAACC-AATTT-------TATTA-GTTGGGGAAT 438
Caulophyllum_thalictroides     CAGACAGCGTCGCCCCCACCCCAACGTGCACACGCACCACAGGGAGGGGC 452
                                **    * ***  ** * ** **  *           *       *

Actaea_dahurica                GGAAATTGGCCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_cimicifuga              GGAAATTGGCCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 475
Actaea_rubra                   GGAAATTGGCCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 460
Actaea_pachypoda               GGAAATTGGCCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTCAAATATTG 461
Actaea_racemosa                GGAAATTGGCCCCCCCGAGTCCTCTTTTGGGCACGGTTGGCTTAAATATTG 519
Actaea_cordifolia              GGAAATTGGCCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 456
Actaea_podocarpa               GGAAATTGGCCCTCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 458
Actaea_heracleifolia           GGAAATTGGCCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTG 457
Actaea_simplex                 GGAGATTGGCCCCCCCGAGTCC--TTTTGGGCACGGTTGGCTCAAATATTC 486
Caulophyllum_thalictroides     GGAGATTGGCCCCCCCGTG-CCGTAAGCAGGCGCGGTCGGCCCAAAAGTCG 501
                               *** ******** *** * **       *** **** ***  ***  *

Actaea_dahurica                GTCCTCGACGGCAAGTGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 523
Actaea_cimicifuga              GTCCTCGGCGGCAAGCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 523
Actaea_rubra                   GTCCTTGACGGCAATCGTCGCGGTCTGTGGTGGTTGTAAA--CTCATCCC 508
Actaea_pachypoda               GTCCTTGACGGCAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 509
Actaea_racemosa                GTCCTCGACGACAATCGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 567
Actaea_cordifolia              GTCCTCGATGACAAGTATCGCGGTCTGCGGTGGTTGTAAA--TTCATCCC 504
Actaea_podocarpa               GTCCTCGRCGGCAAGTGTCGTGGTCTGCGGTGGTTGTAAA--CTCATCCC 506
Actaea_heracleifolia           GTCCTCGACGGCAAGTGTCGCGGTCTGCGGTGGTTGTAAA--CTCATCCC 505
Actaea_simplex                 GTCCTCGGCGGCAAGTGTTGCGGTCTACGGTGGTTGTAAA--CTCATCCC 534
Caulophyllum_thalictroides     ACCCTCGGCGACGAGCGTCACGATCATTGGTGGTTGAGAAGCCCCCTCGT 551
                                 *** *  * * *   *   * **   ********  **    * **

Actaea_dahurica                CCTAAGACAAAATAAGACGCRTAGACTTGTCGTCTAAA-GGACCAACATA 572
Actaea_cimicifuga              CCTAAGACGAAATAAGACGCGTAGCCTTGTCGTATAAY-GGACCAACATA 572
Actaea_rubra                   CCTAAGACGAAATAAGACGCGTAGCCTTGTTGTCTAAT-AGACCAACATA 557
Actaea_pachypoda               CCTAAGACGAAATAAGACGCGTAGCCTTGTTGTCTAAT-AGACCAACATA 558
Actaea_racemosa                CCTAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAT-AGACCAACATA 616
Actaea_cordifolia              CCCAAGACGAAATAAGACGTGTAGCCTTGTTGTCTAAT-GGACCAACATA 553
Actaea_podocarpa               CCTAAGACGAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACCAACATA 555
```


Actaea_heracleifolia C-TAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACCAACATA 553
Actaea_simplex CCTAAGACAAAATAAGACGCGTAGCCTTGTCGTCTAAC-GGACTAACATA 583
Caulophyllum_thalictroides CGTAGACCGGCGT----CGTGGCGCCTCGTCGCCTTACCGGGTCGGAAGA 597
Actaea_dahurica ACCCTTGGAAGCCGTTCAACGGTGTTCACCCT---------- 604
Actaea_cimicifuga ACCCTTRGAAGCCGTTCAACGGTTTTCACCCT---------- 604
Actaea_rubra ACCCTTGGAAGCCGTTTAACGGTGTTCACCTTGCGACCCCAGGTCAGGCG 607
Actaea_pachypoda ACCCTTGGAAGCCGTTTAACGGTGTTCACCTTGCGACCCCAGGTCAGGCG 608
Actaea_racemosa ACCCTTGGAAGCCGTTTAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 666
Actaea_cordifolia ACCCTTTGAAGCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 603
Actaea_podocarpa CCCCTTATAATCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 605
Actaea_heracleifolia ACCCTTGGAAGCCGTTCAACGGTGTTCACCCTGCGACCCCAGGTCAGGCG 603
Actaea_simplex ACCCTTGGAAGCCGTTCATCGGTGTTCACCCTGCGACCCCAGGTCAGGCG 633
Caulophyllum_thalictroides ACCCTC----------GTTTGTCCTGCA---------- 615

8.3 nrITS sequence traces of samples used in the development of PlantID

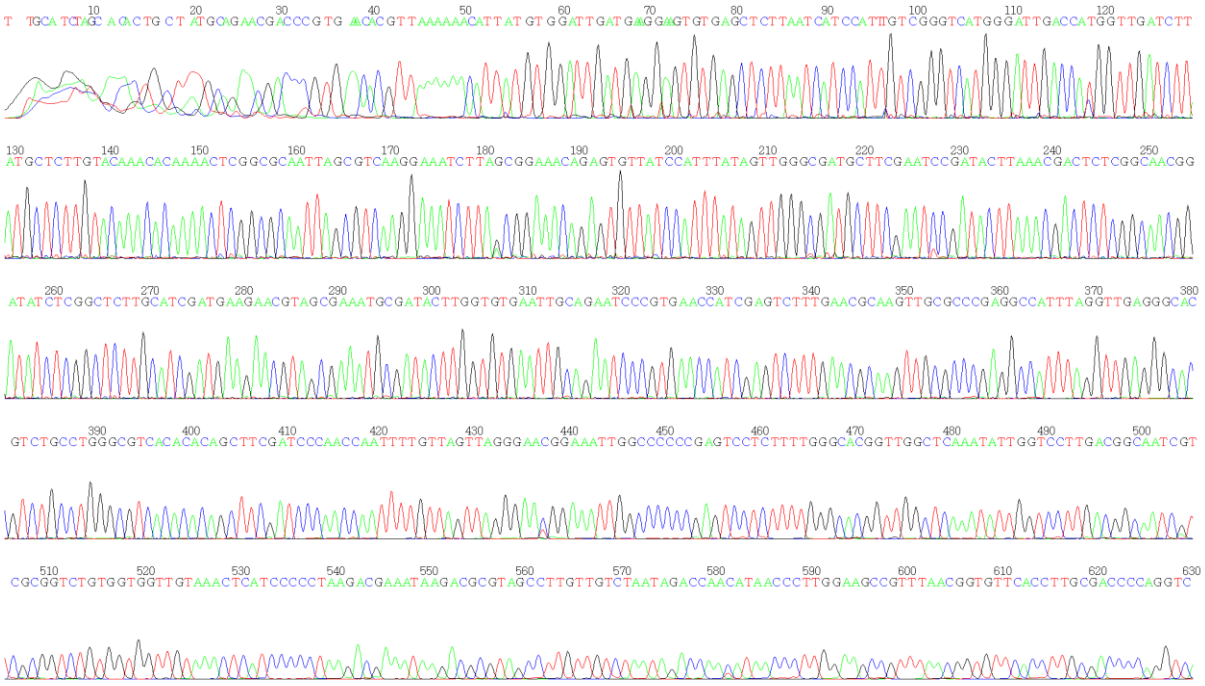

Figure 75: Sequence trace of *Actaea rubra* – DNA extracted from material supplied by CAMAG, Switzerland

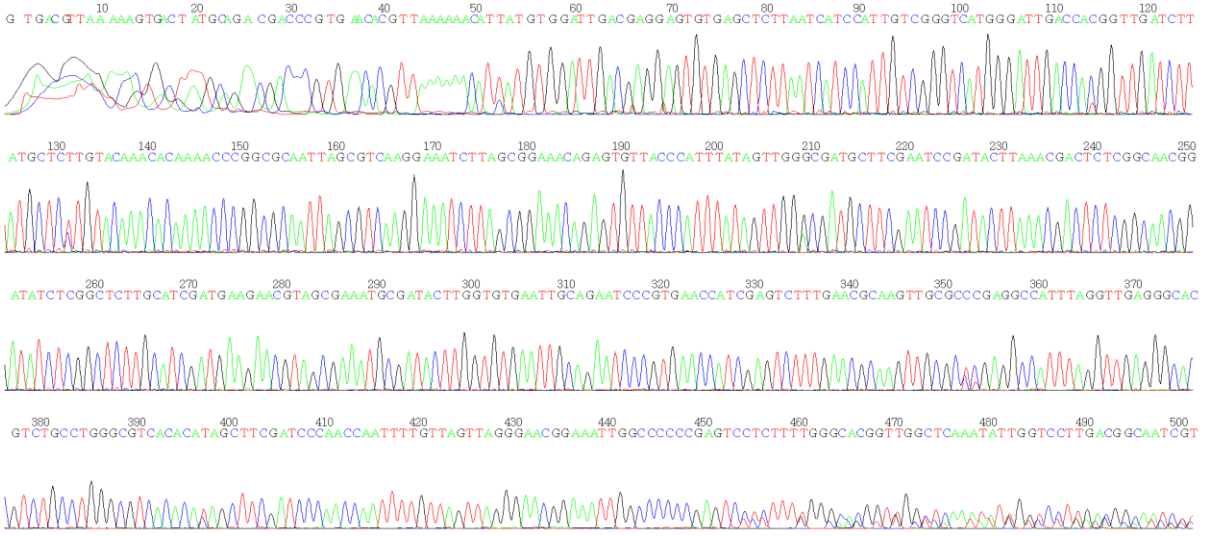

Figure 76: Sequence trace of *Actaea pachypoda* – DNA extract supplied by Royal Botanical Gardens, Kew

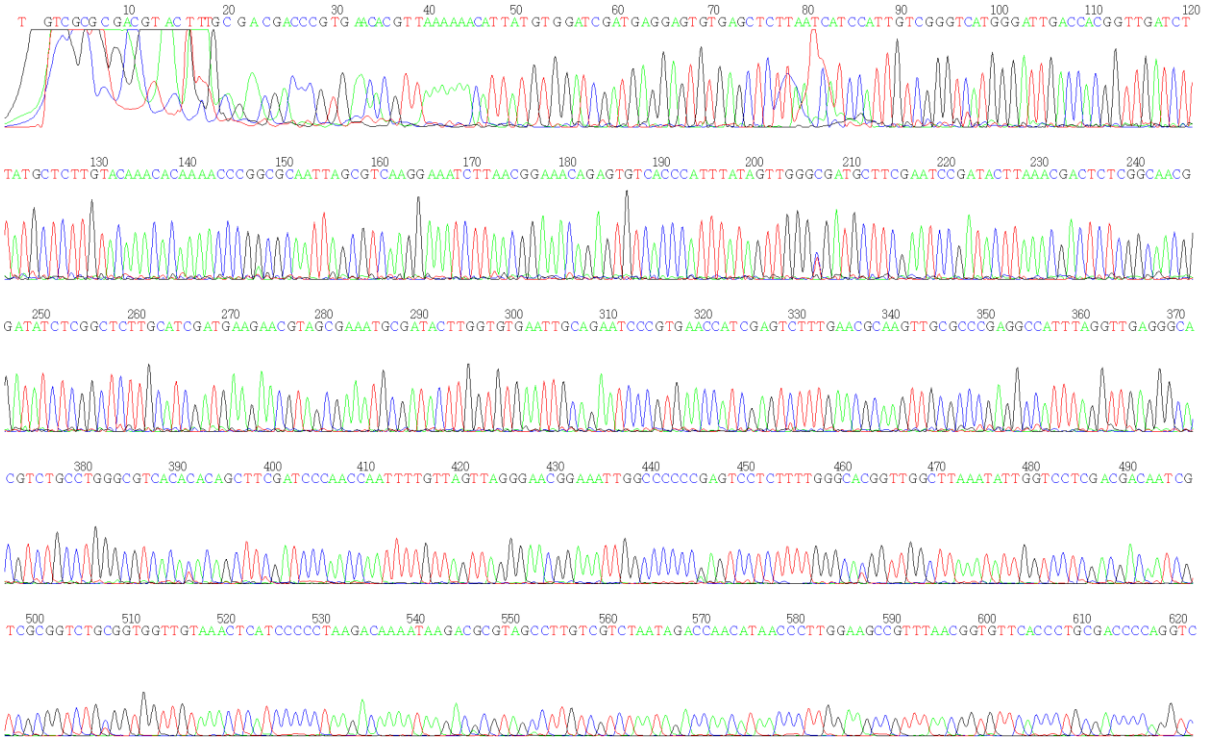

Figure 77: Sequence trace of *Actaea racemosa*– DNA extract supplied by Royal Botanical Gardens, Kew

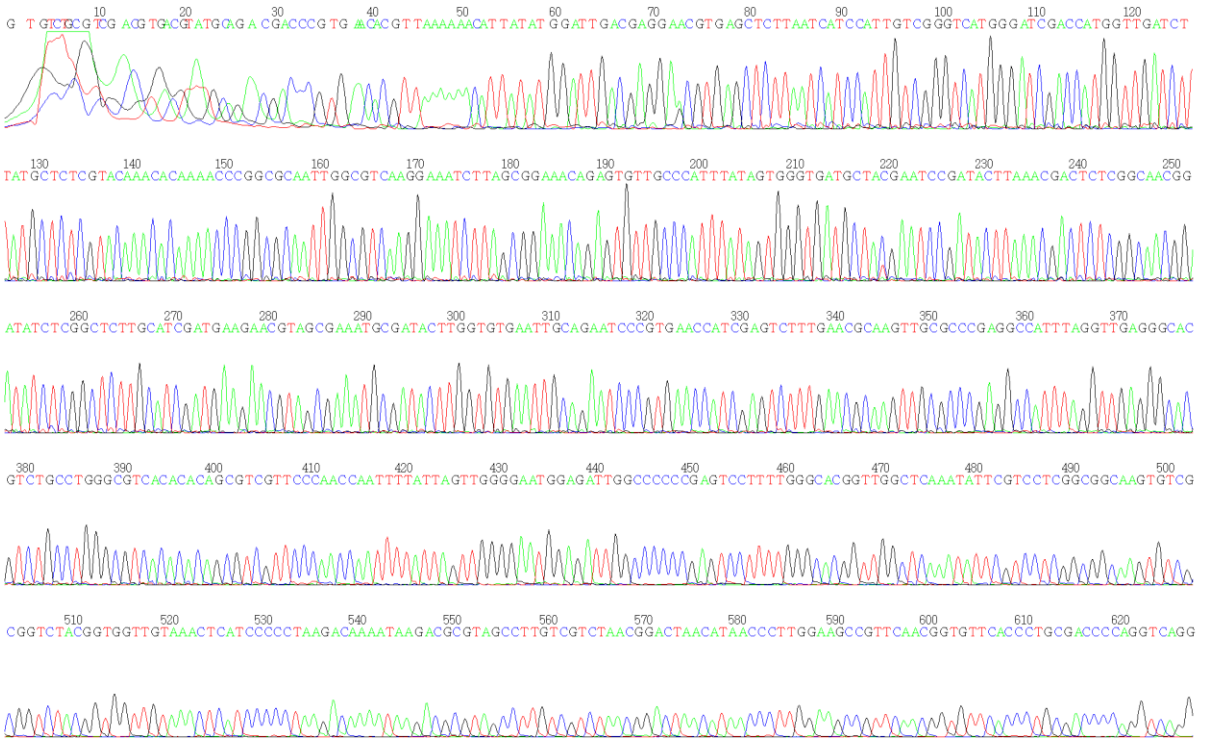

Figure 78: Sequence trace of *Actaea dahurica,* identified as *Actaea simplex* – from Royal, Botanical Gardens, Kew

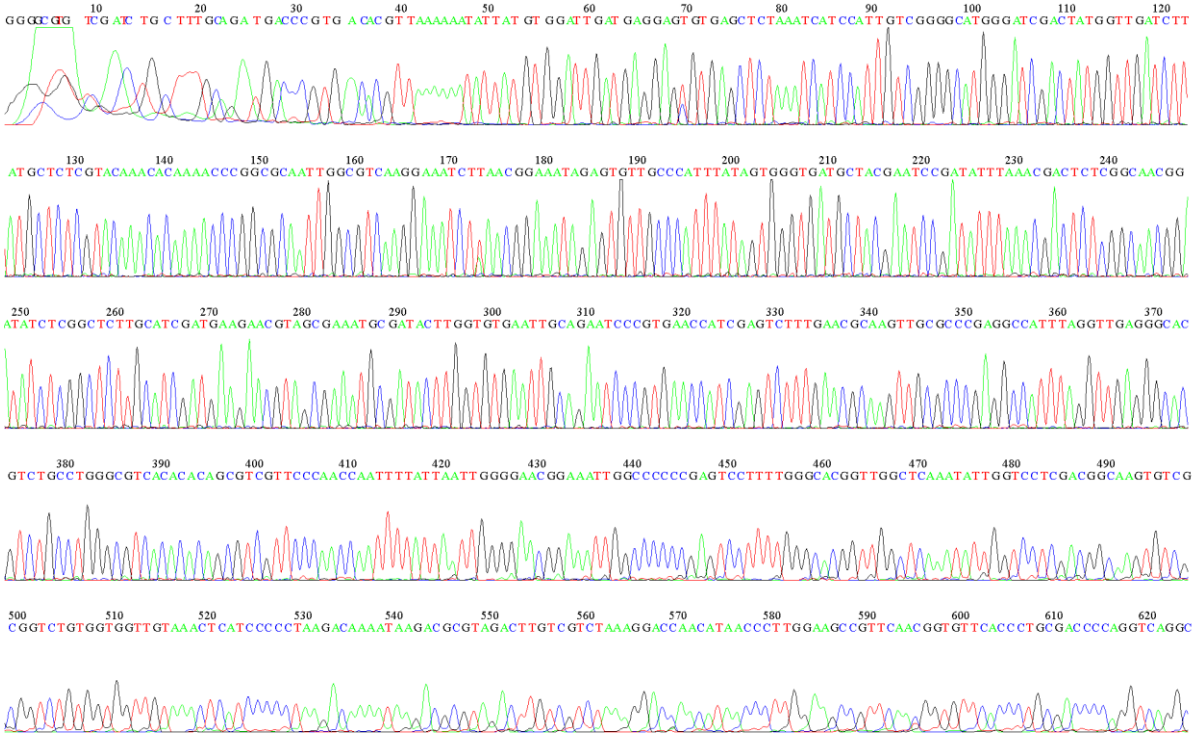

Figure 79: Sequence trace of *Actaea dahurica* from Botanical Gardens and Botanical Museum, Germany

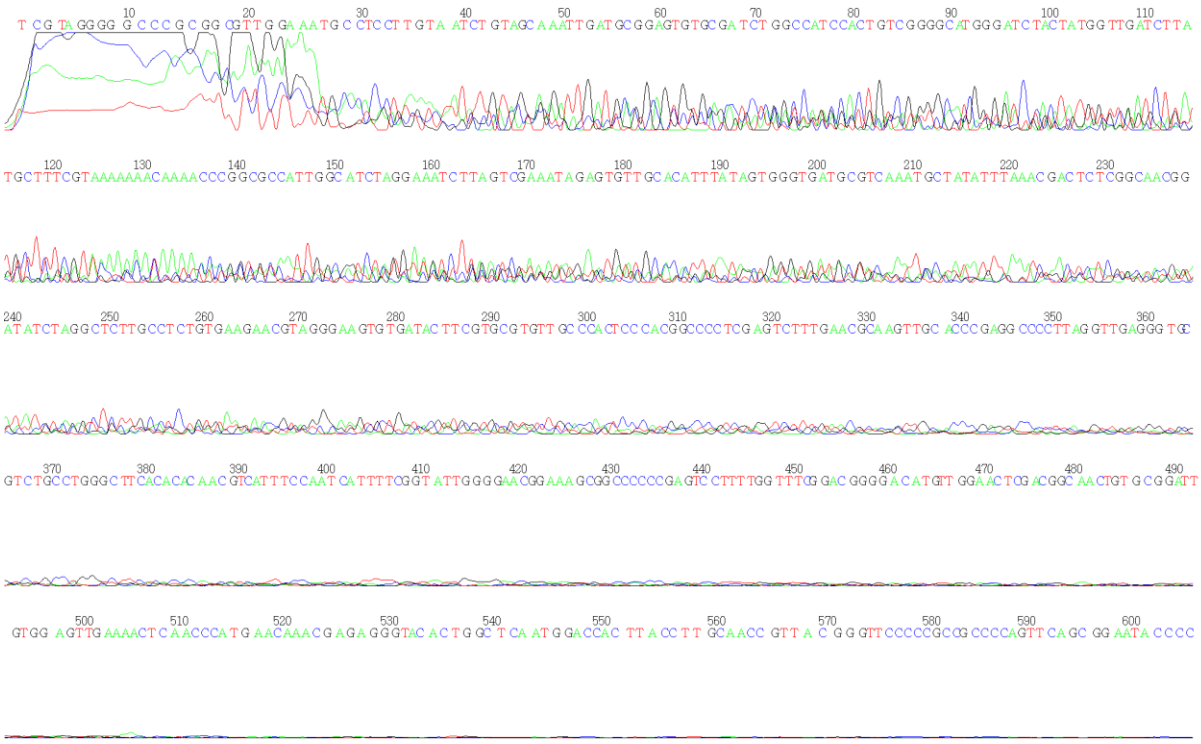

Figure 80: Sequence trace of *Actaea heracleifolia* – DNA extract from material supplied by CAMAG, Switzerland

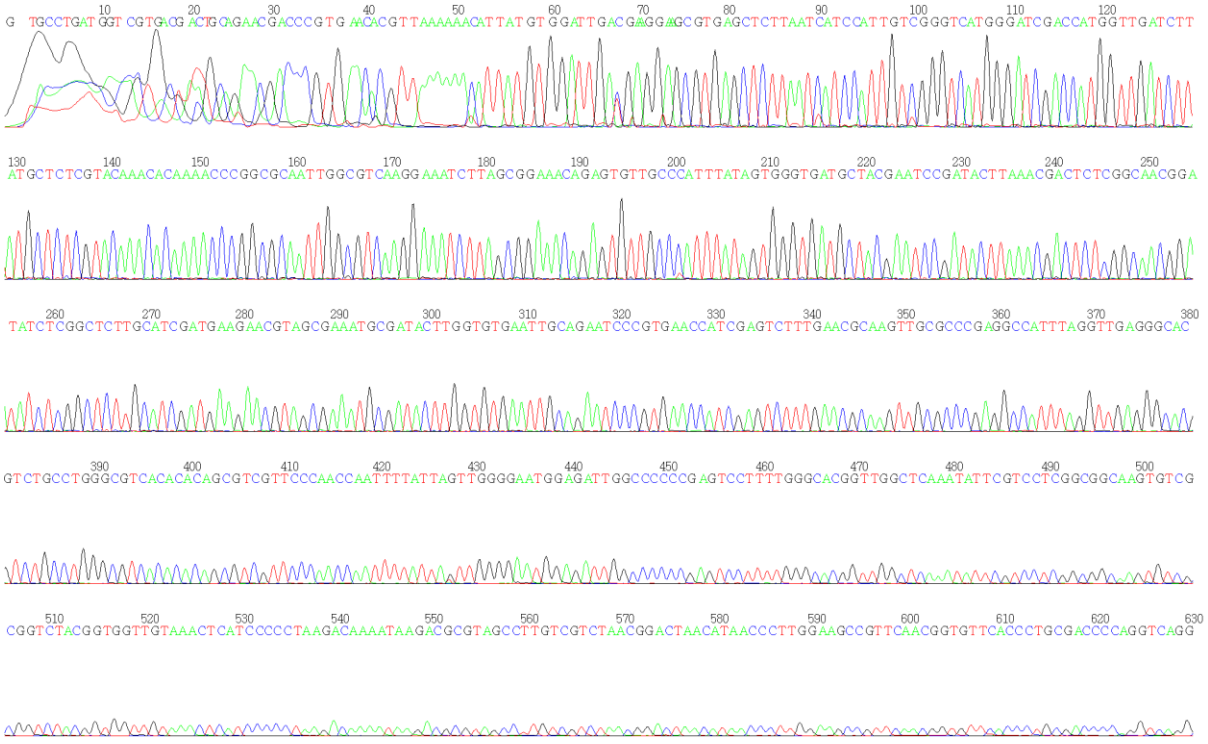

Figure 81: Sequence trace of *Actaea simplex* – DNA extract from plant material provided by Beeches Nursery

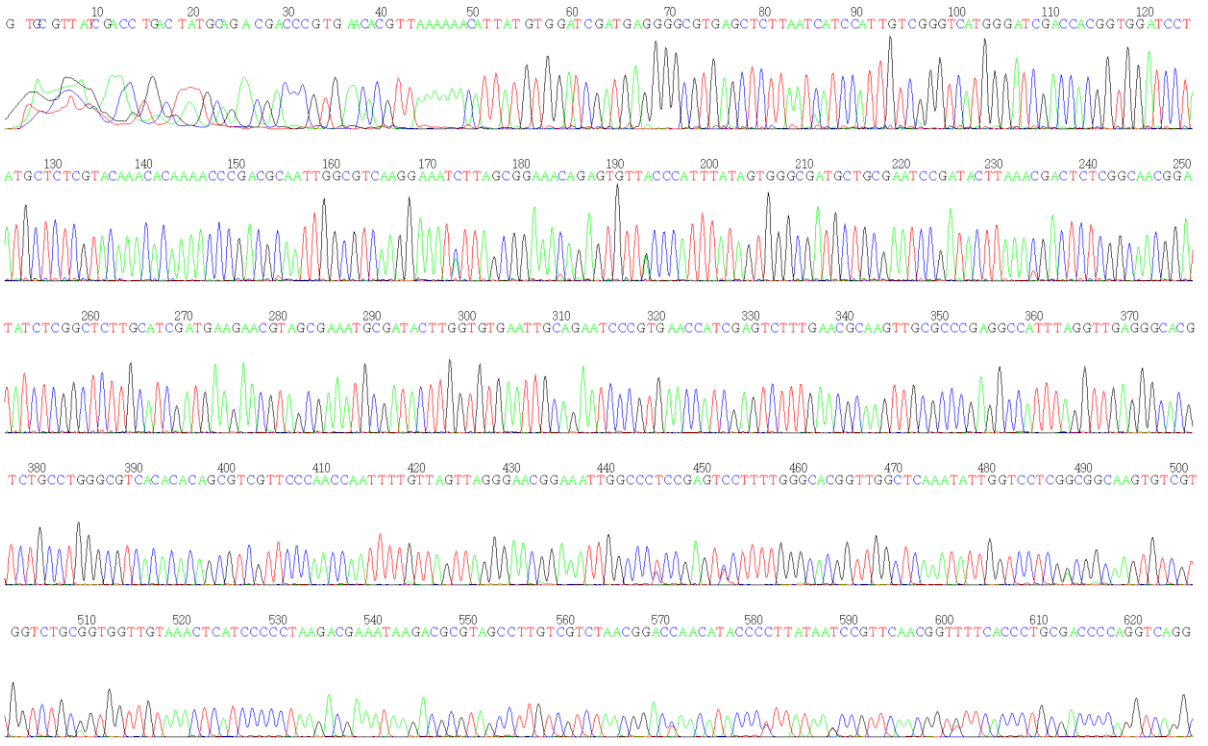

Figure 82: Sequence trace of *Actaea podocarpa* – DNA extract from material supplied by CAMAG, Switzerland

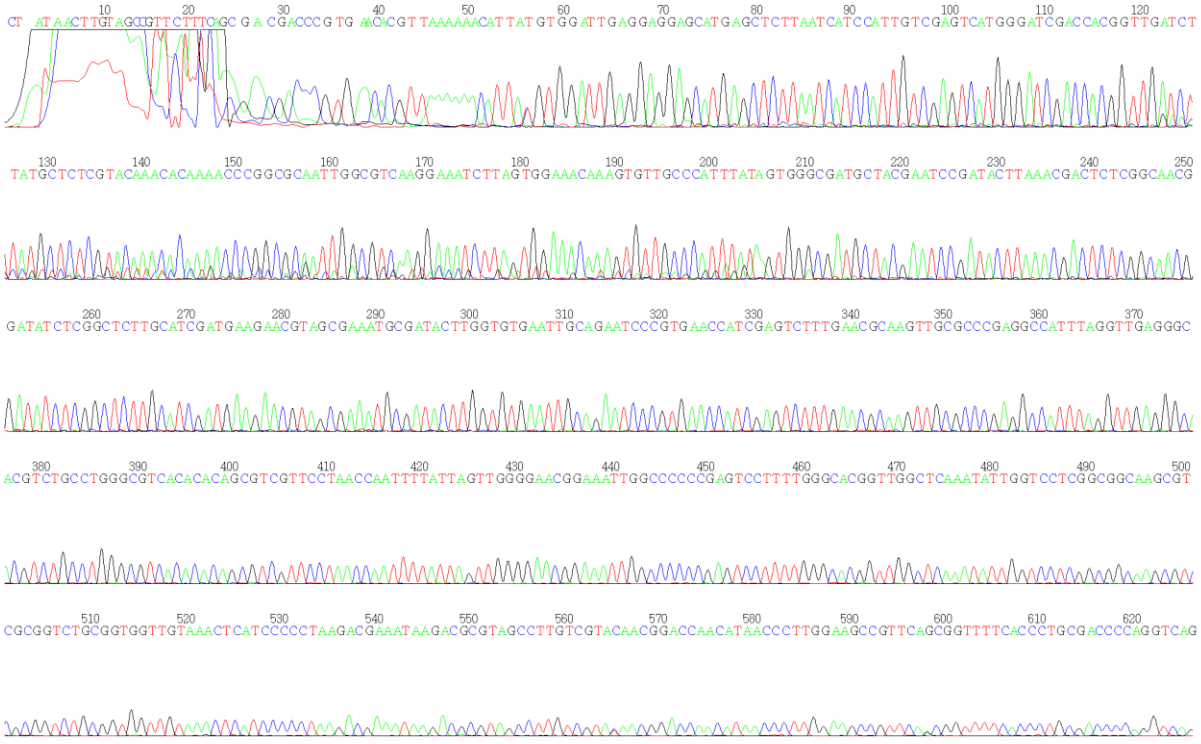

Figure 83: Sequence trace of *Actaea cimicifuga* – DNA extract from Royal Botanical Gardens, Kew

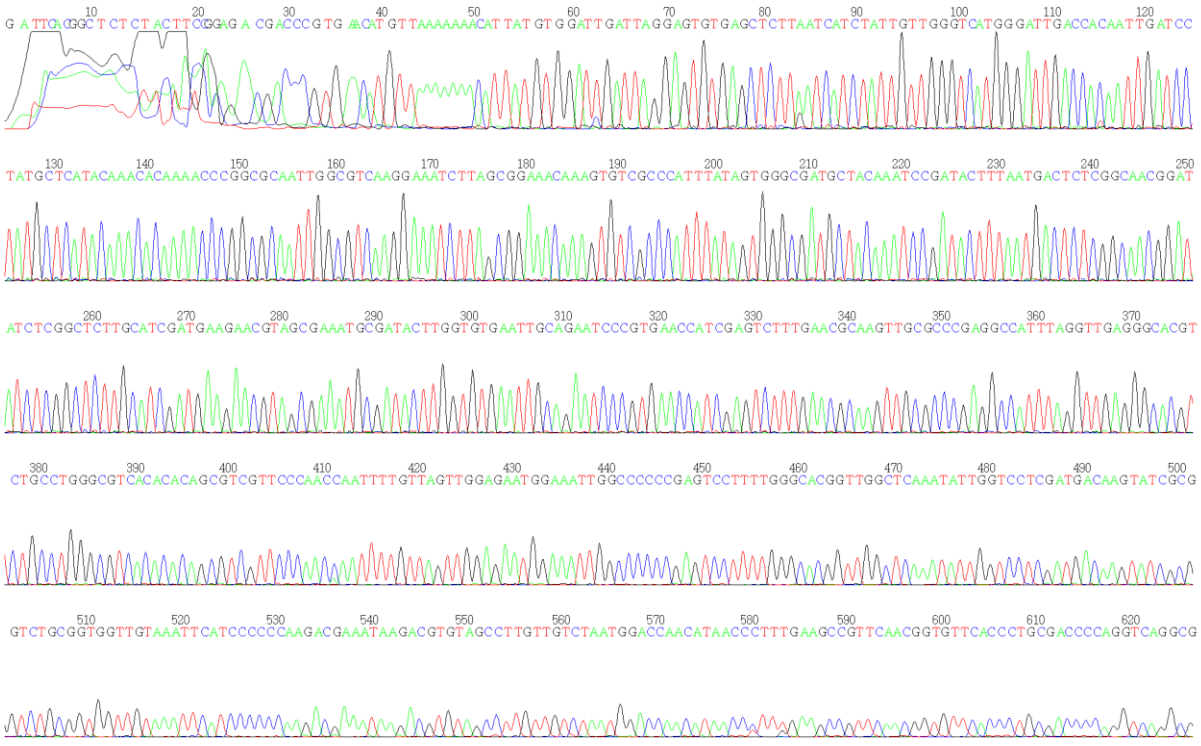

Figure 84: Sequence trace of *Actaea cordifolia* – DNA extract supplied by Royal Botanical Gardens, Kew

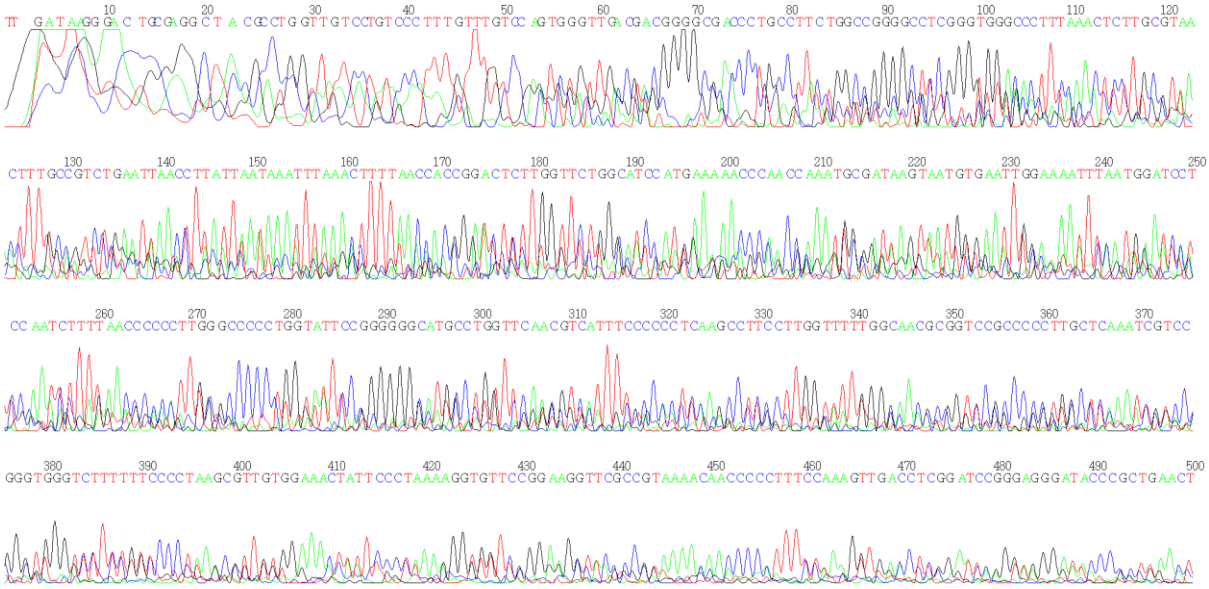

Figure 85: Sequence trace of *Caulophyllum thalictroides* – DNA extract supplied by Royal Botanical Gardens, Kew

8.4 Sequence trace information for reference and test samples

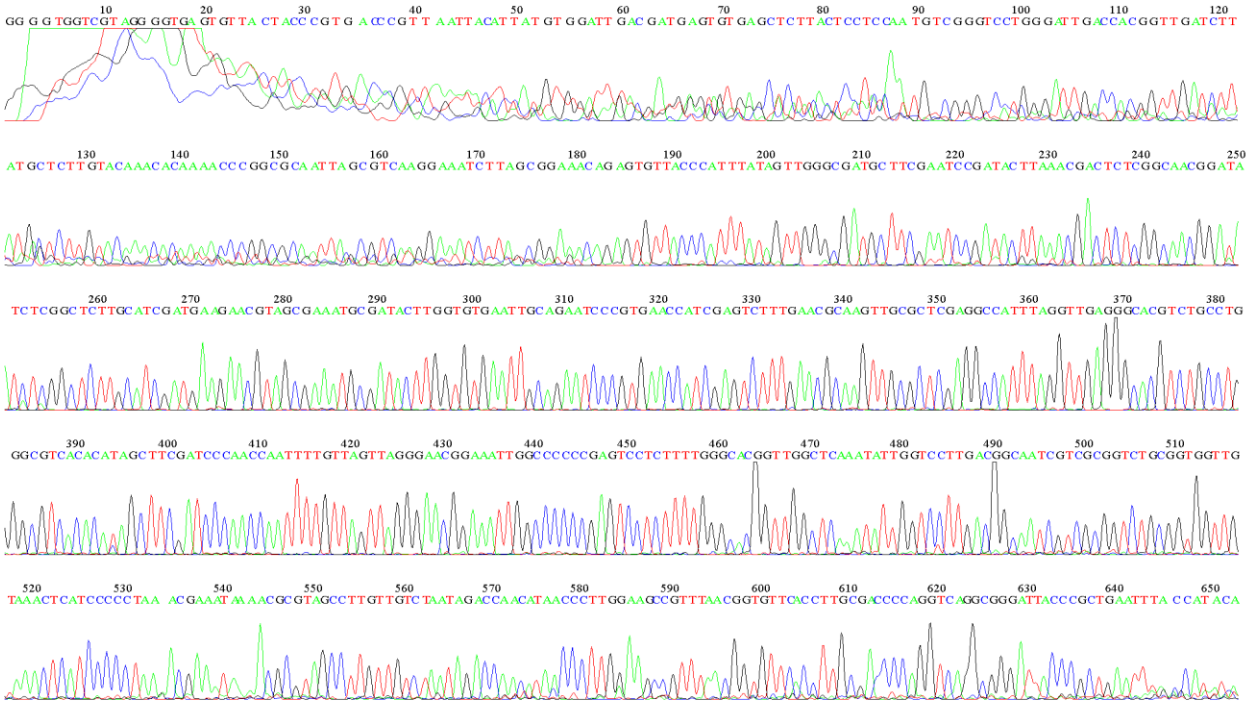

Figure 86: *Actaea pachypoda* –nrITS - BGBM 8495

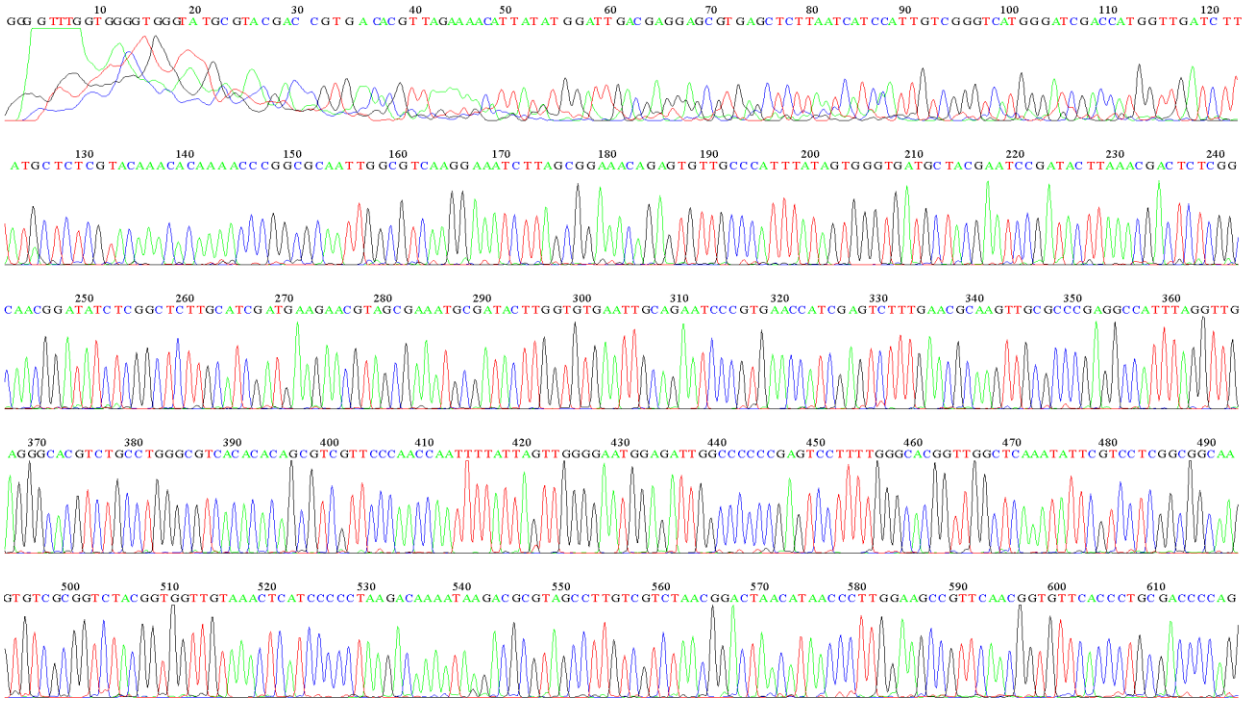

Figure 87: nrITS - *Actaea simplex* – BGBM 8498

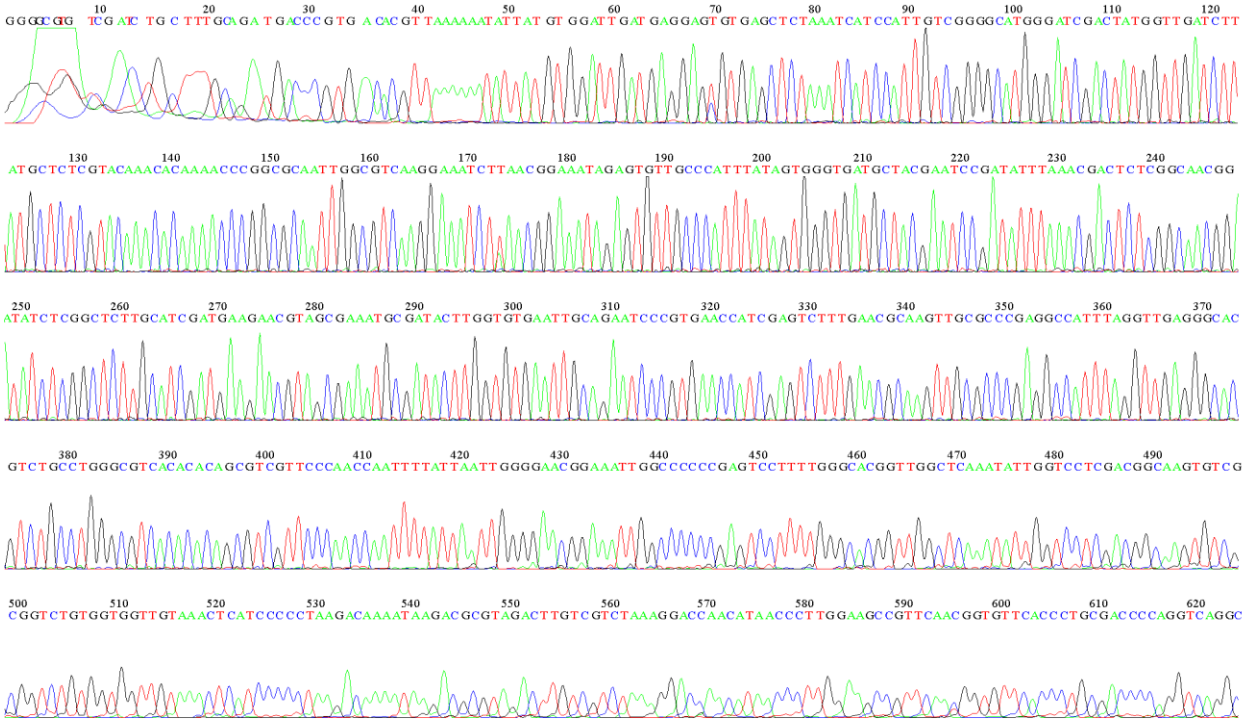

Figure 88: nrITS - *Actaea dahurica* – BGBM 8499

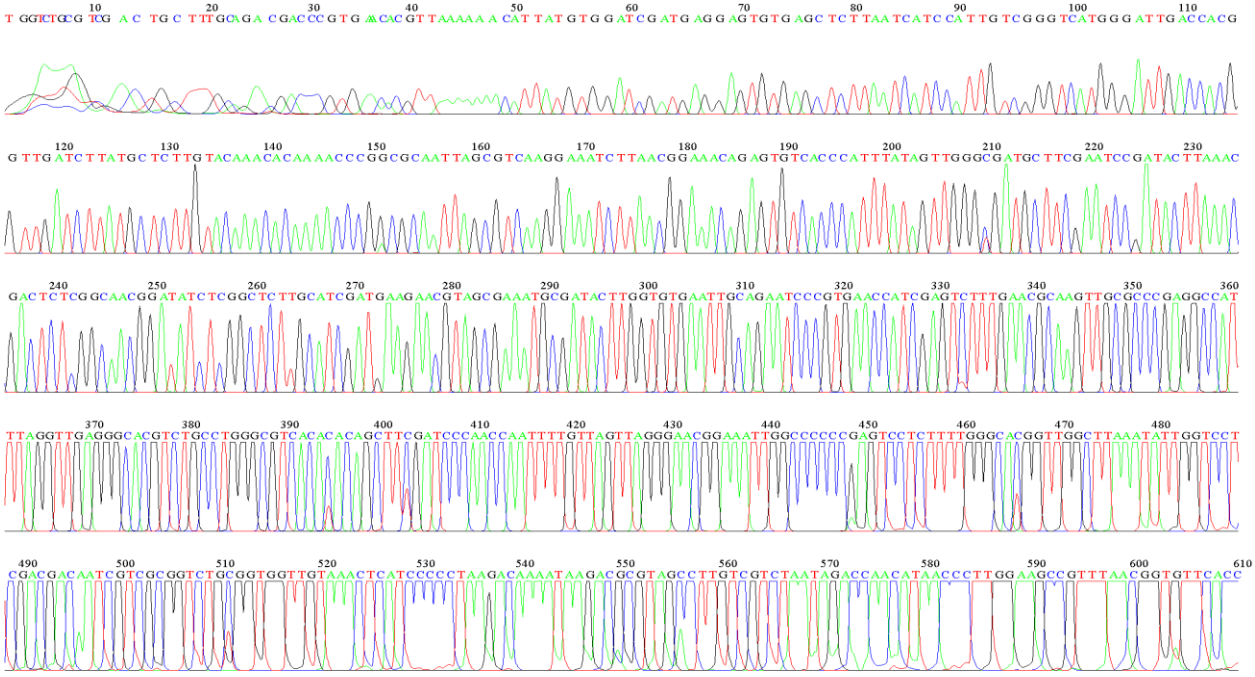

Figure 89: 067 - nrITS - *Actaea racemosa* – Secret Seeds

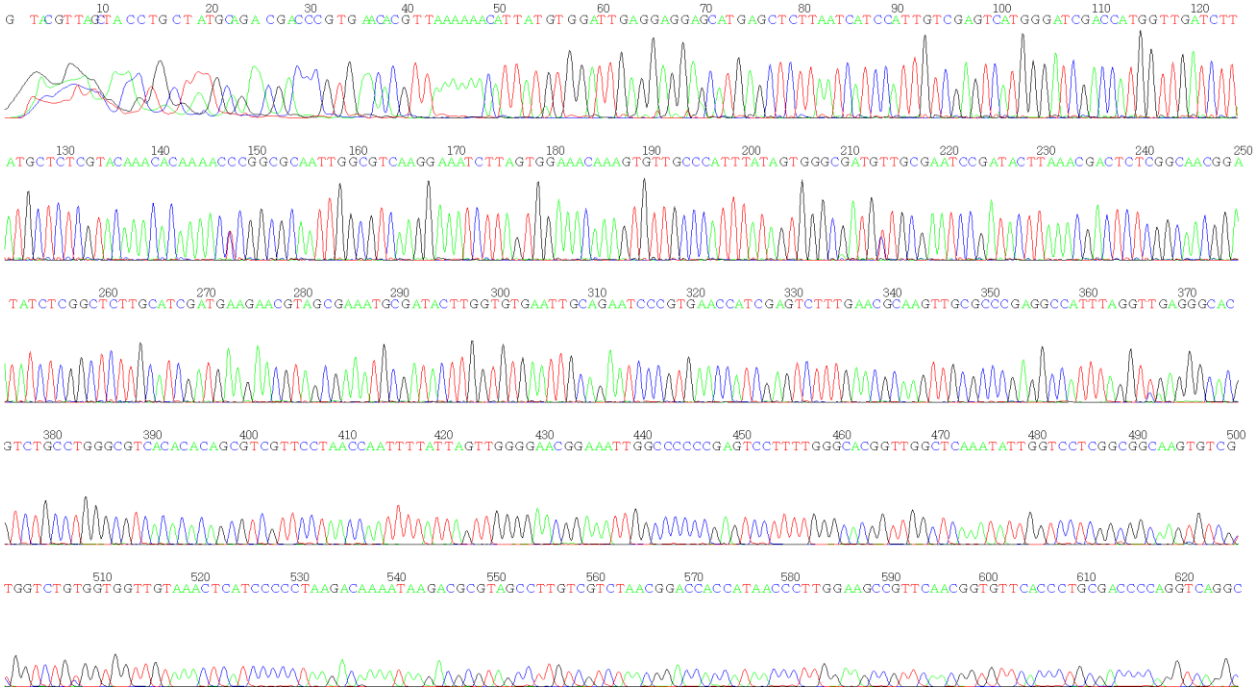

Figure 90: 068 – nrITS - *Actaea cimicifuga* – Secret Seeds

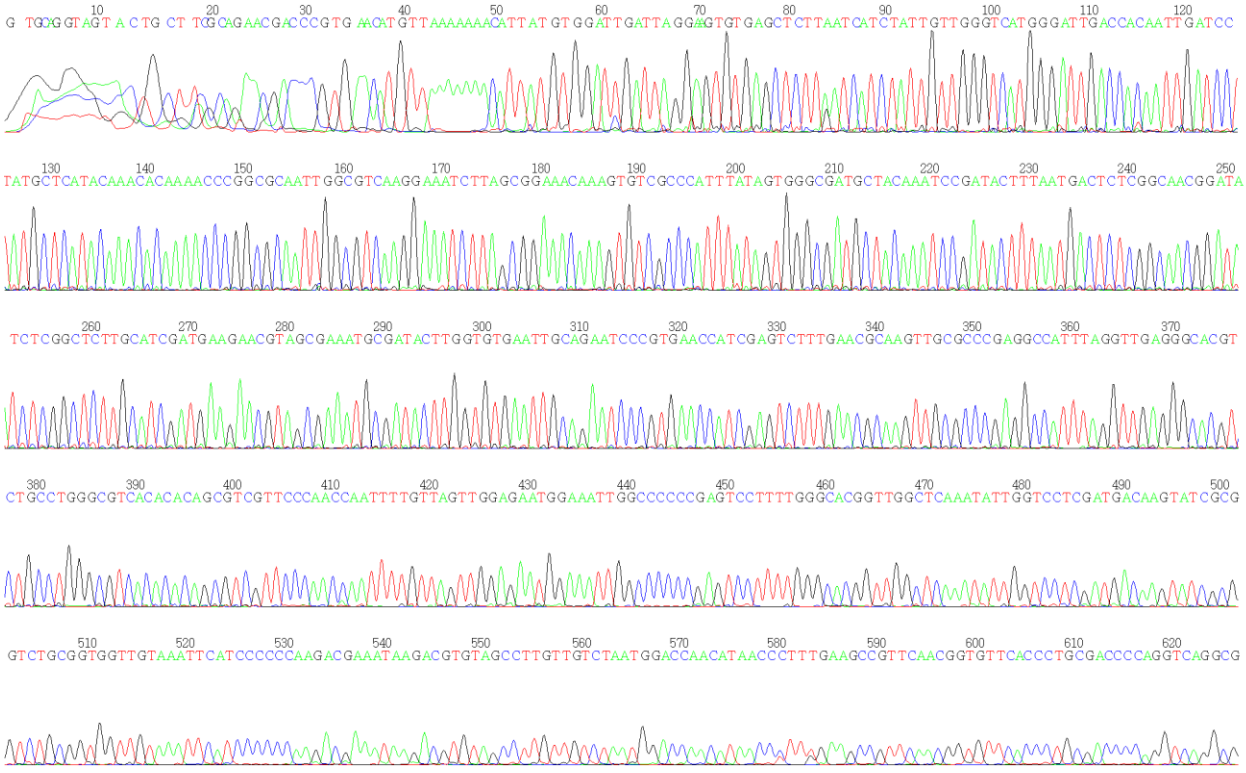

Figure 91: 069 - nrITS -– *Actaea cordifolia* – Secret Seeds

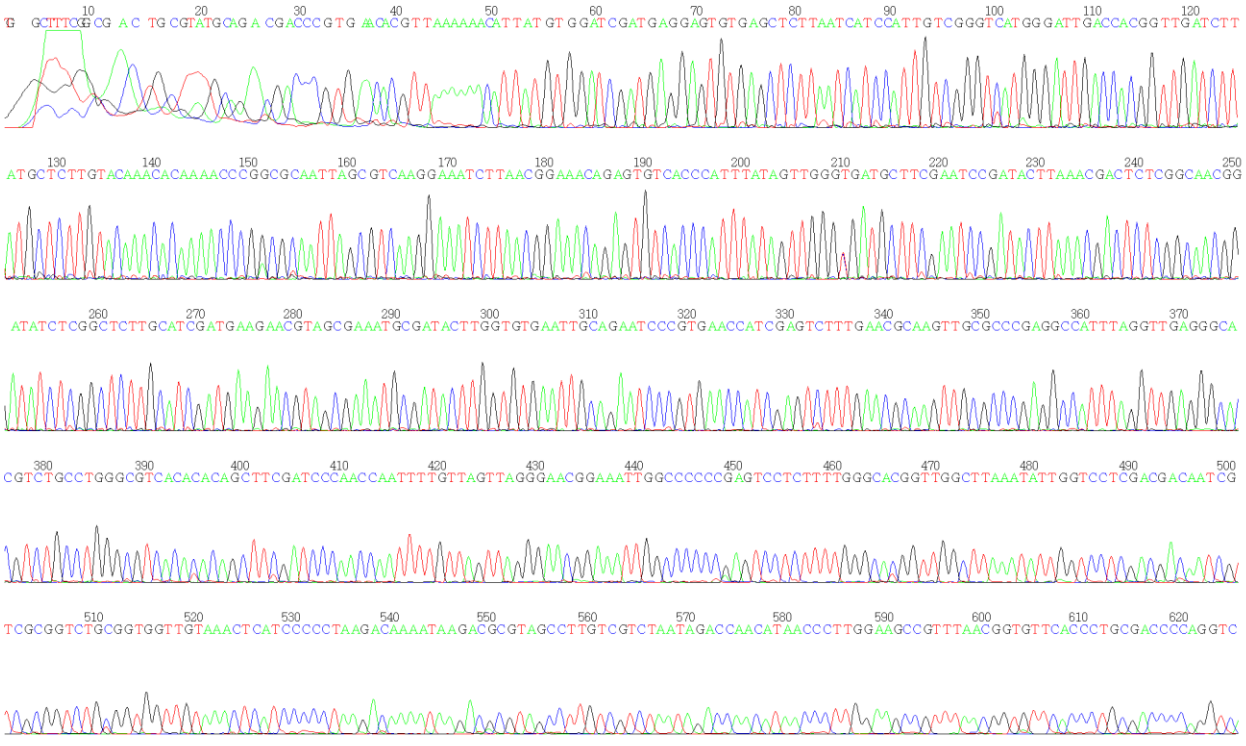

Figure 92: 070 – nrITS - *Actaea racemosa* –Secret Seeds

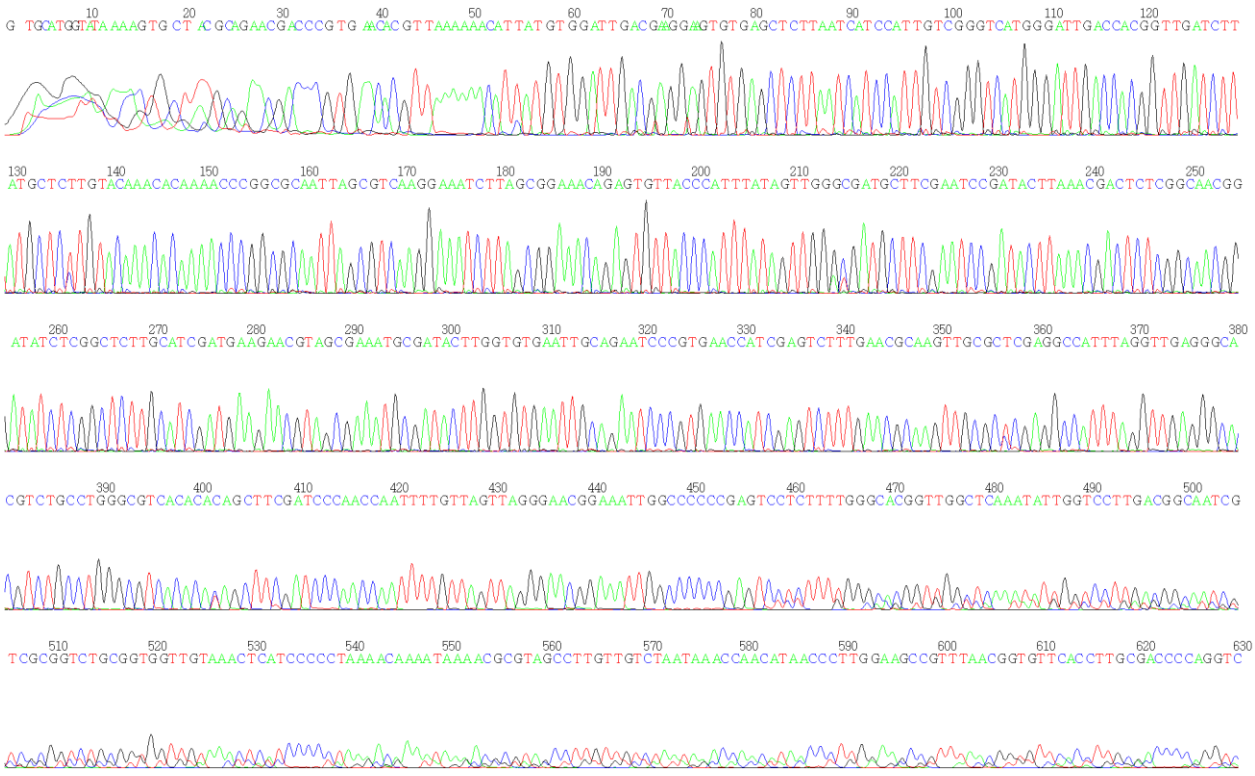

Figure 93: 072 – nrITS - *Actaea pachypoda* –Secret Seeds

Figure 94: S2 – nrITS - *Actaea racemosa* – AHPA-BC014

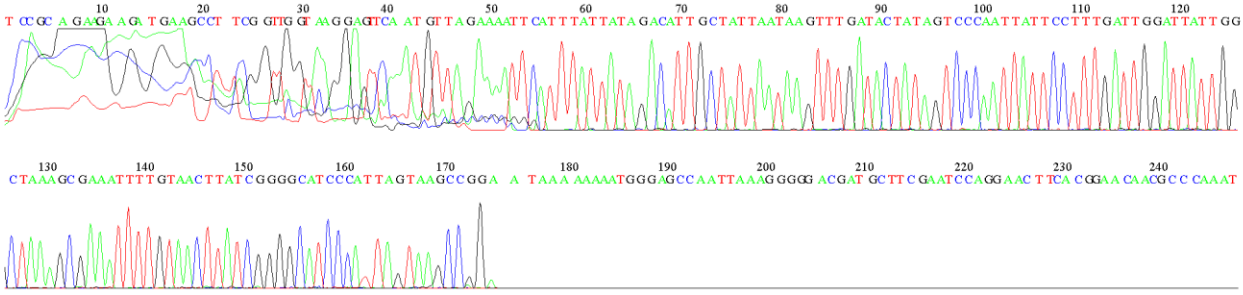

Figure 95: S3 – *matK* – *Actaea racemosa* – AHPA-BC019

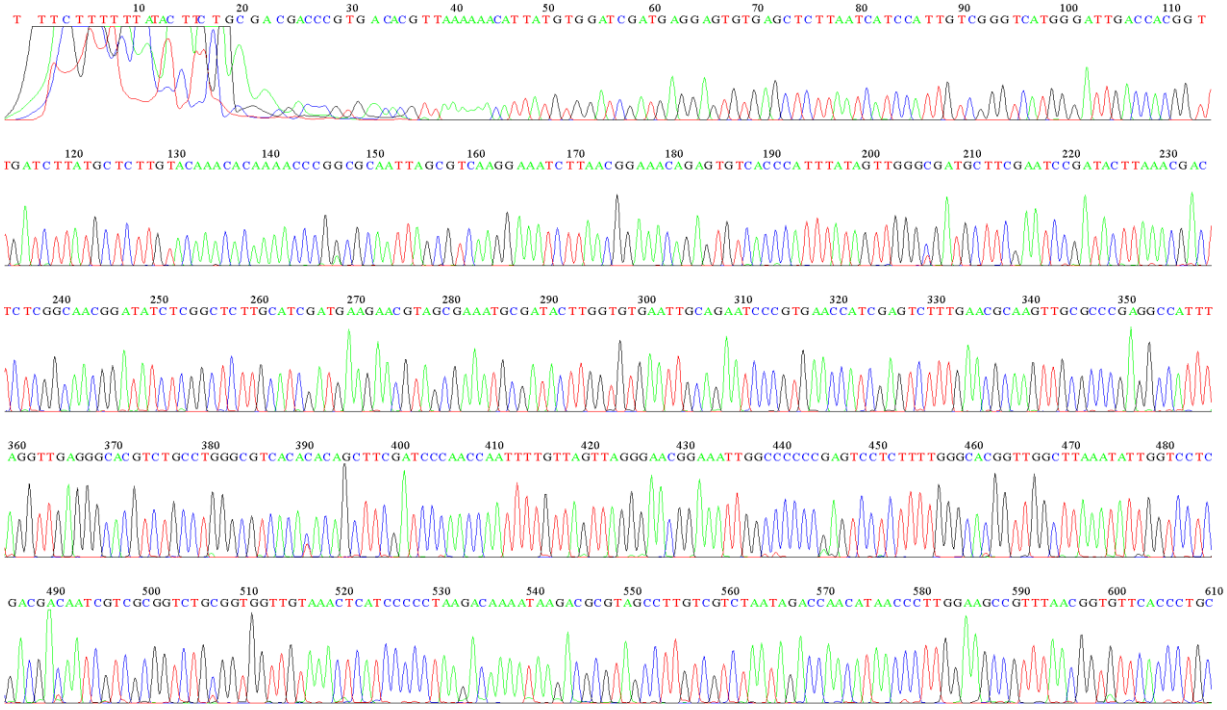

Figure 96: S8 – nrITS – *Actaea racemosa* – AHAP-BC042

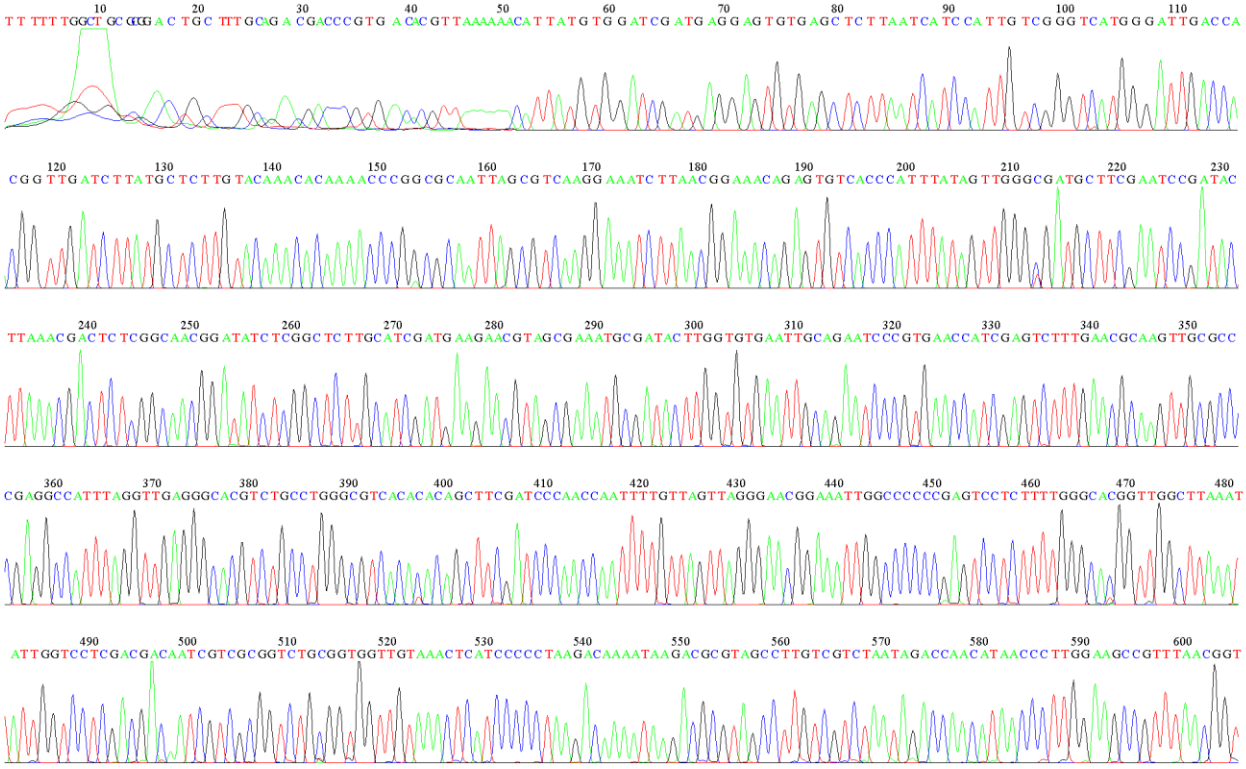

Figure 97: S9 – nrITS – *Actaea racemosa* – AHPA-BC043

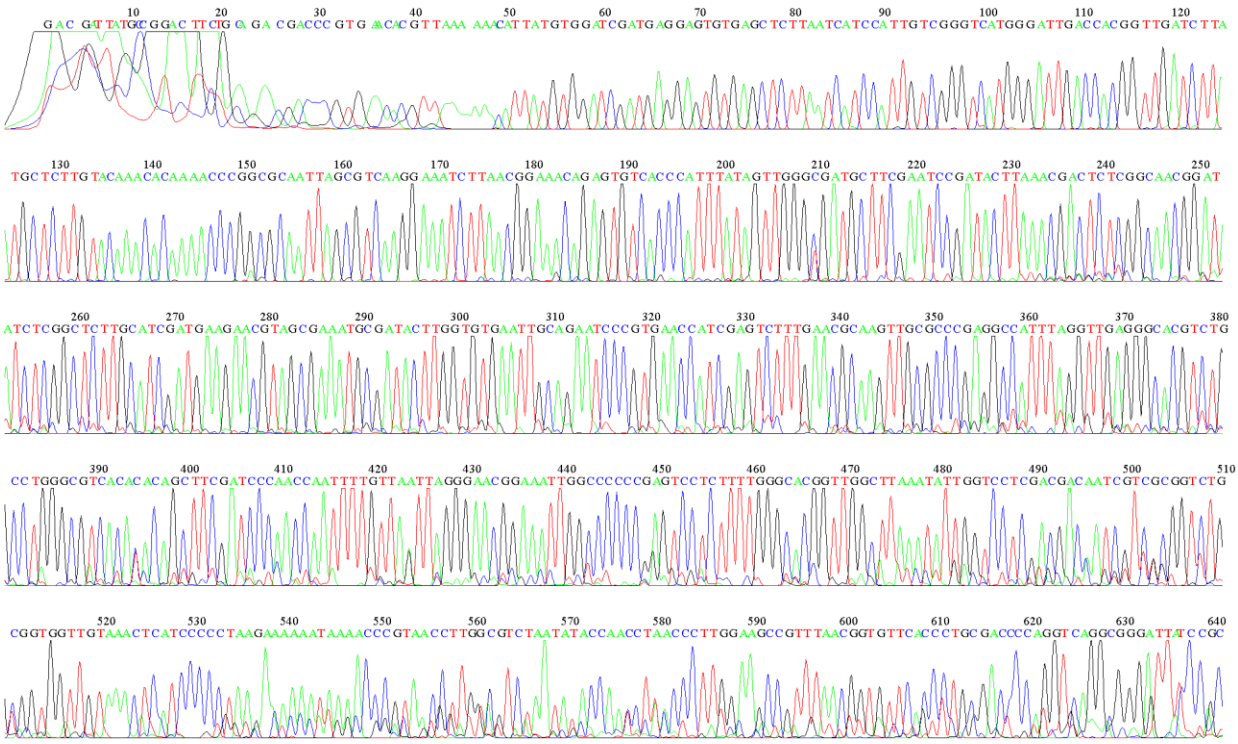

Figure 98: S12 – nrITS – *Actaea racemosa* - AHPA-BC064

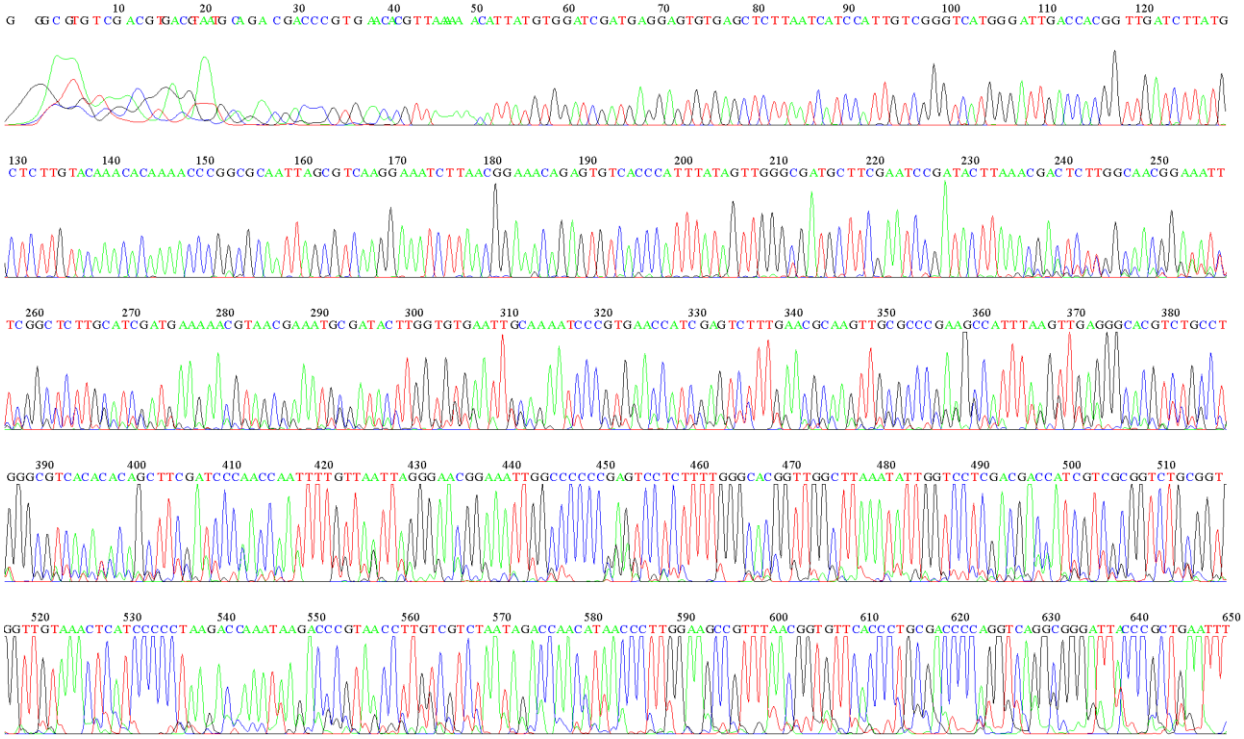

Figure 99: S13 – nrITS – *Actaea racemosa* - AHPA-BC065

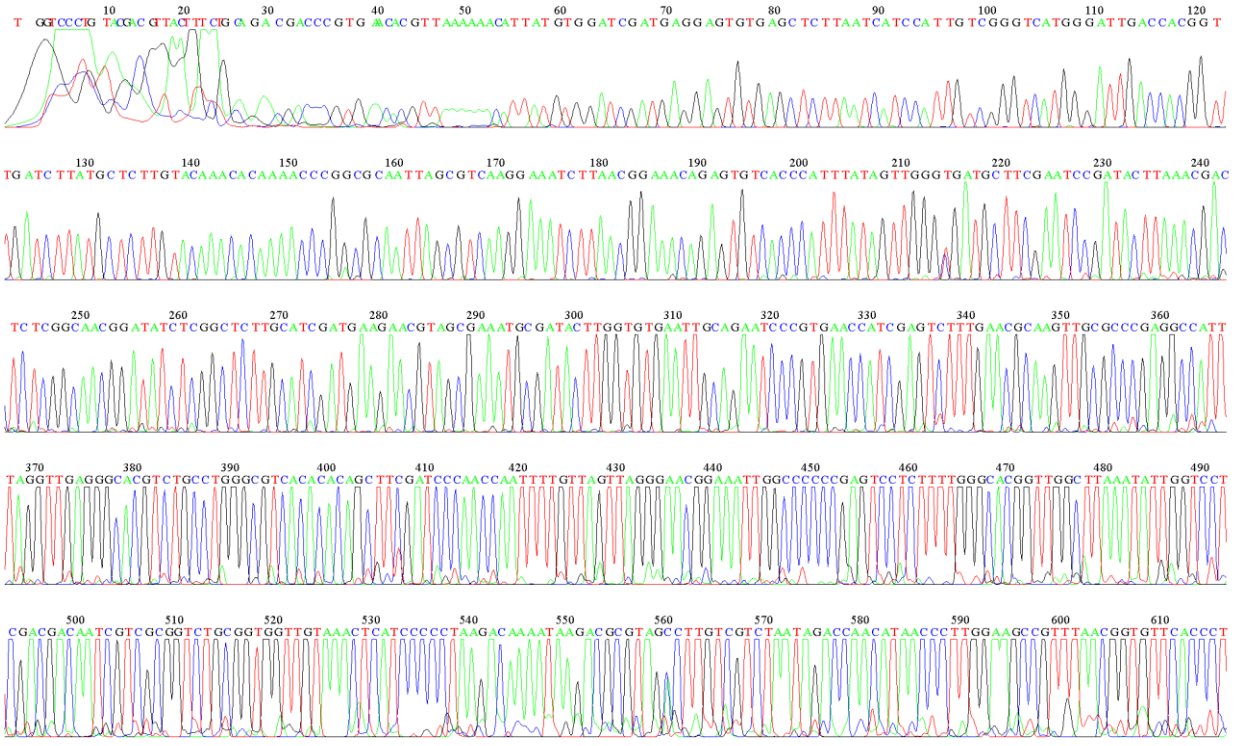

Figure 100: S14 – nrITS – *Actaea racemosa* - AHPA-BC068

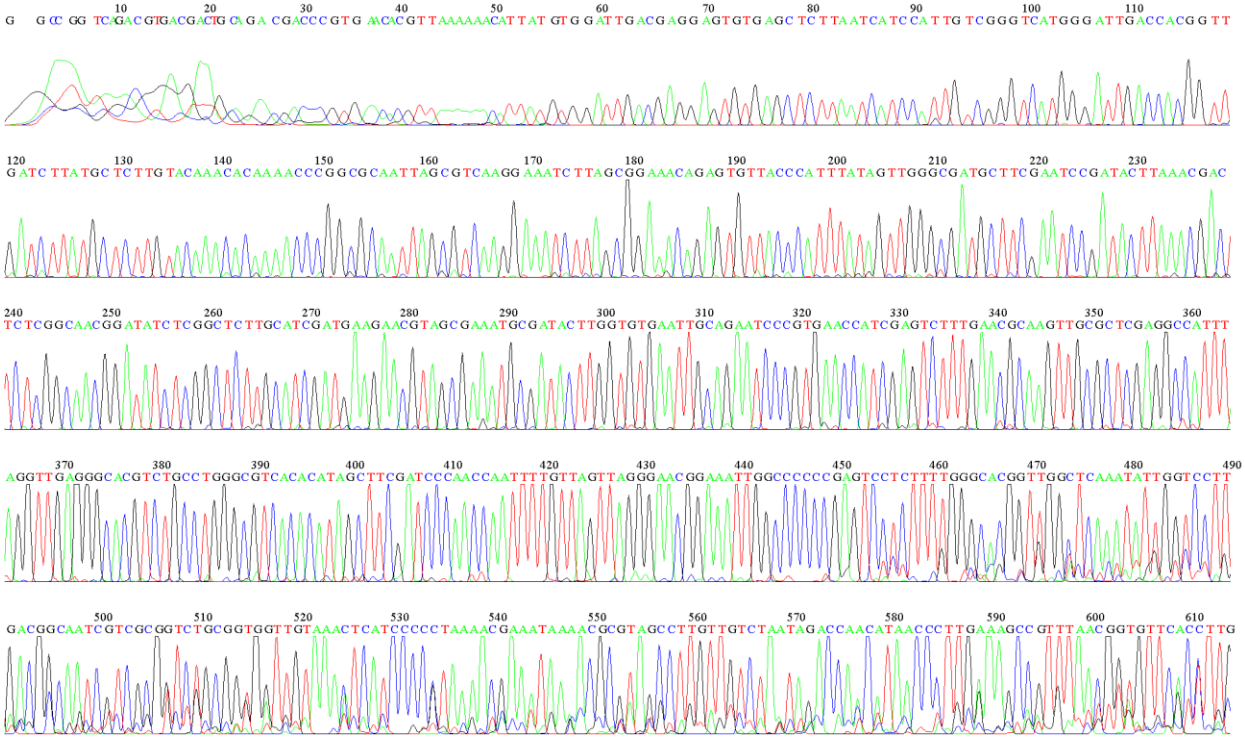

Figure 101: S15 - nrITS – *Actaea racemosa* - AHPA-BC078

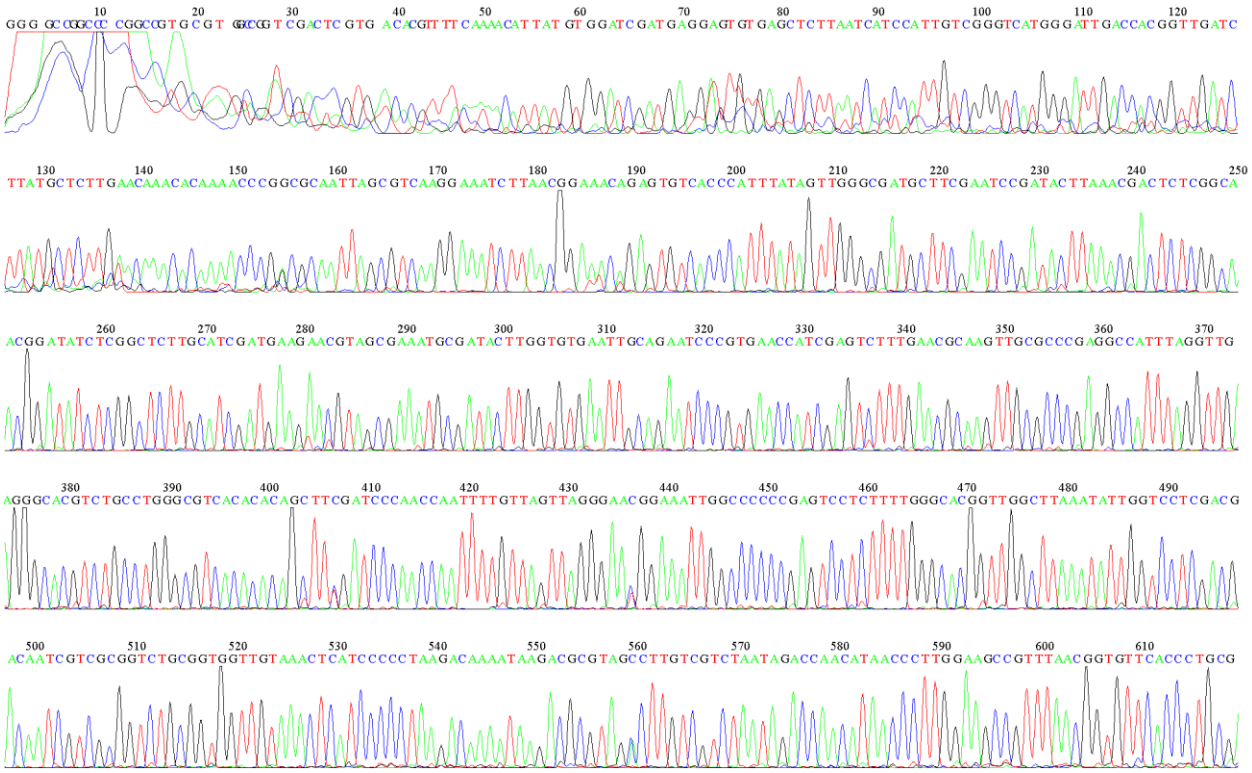

Figure 102: S17 – nrITS – *Actaea racemosa* – AHPA-BC066

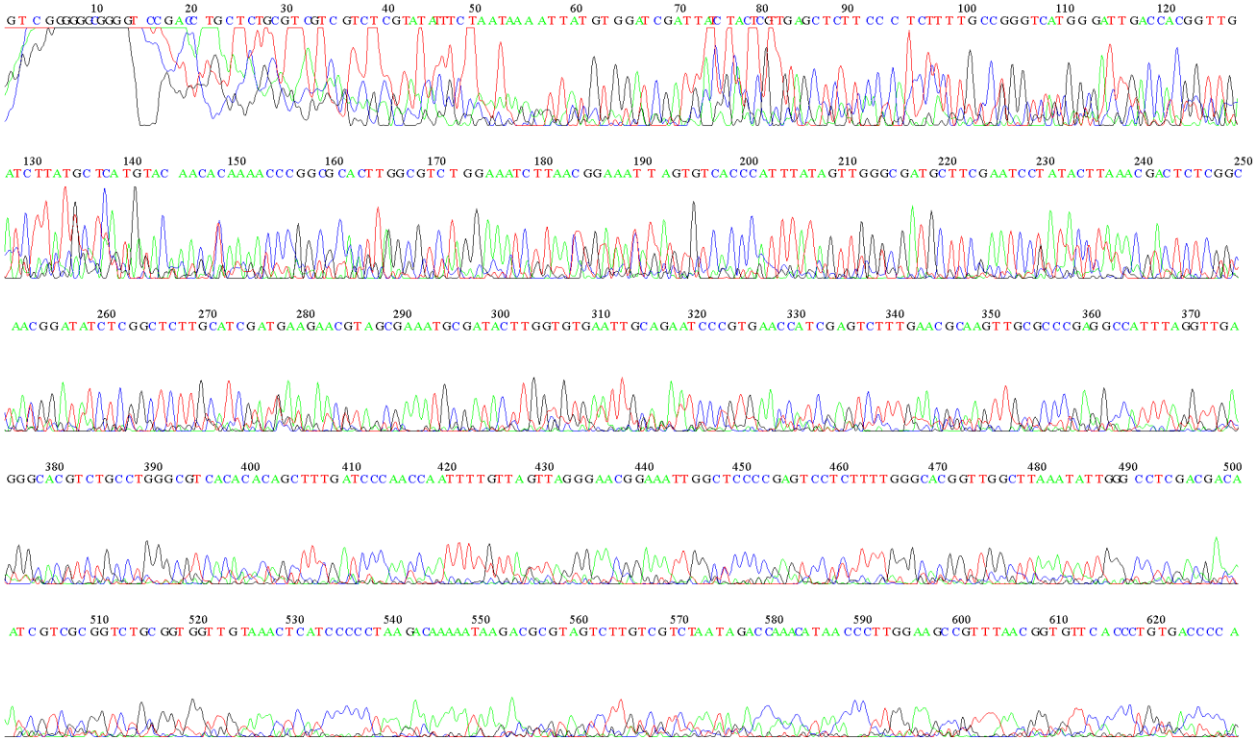

Figure 103: S18 – nrITS – *Actaea racemosa* - AHPA-BC067

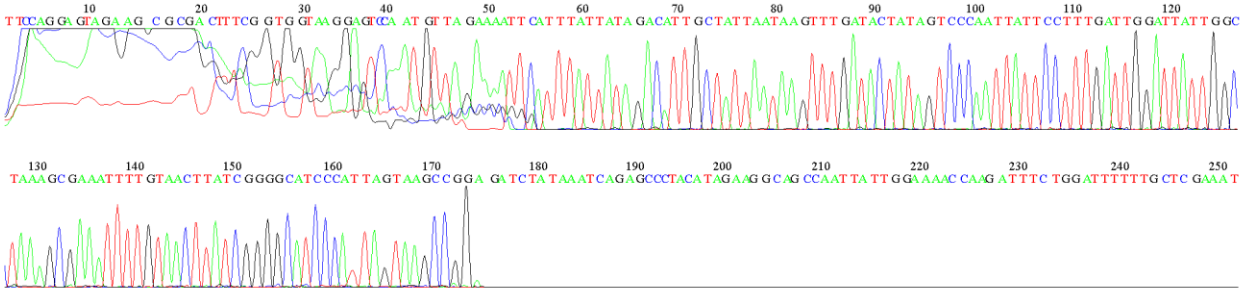

Figure 104: S19 - *matK – Actaea racemosa* – AHPA-BC069

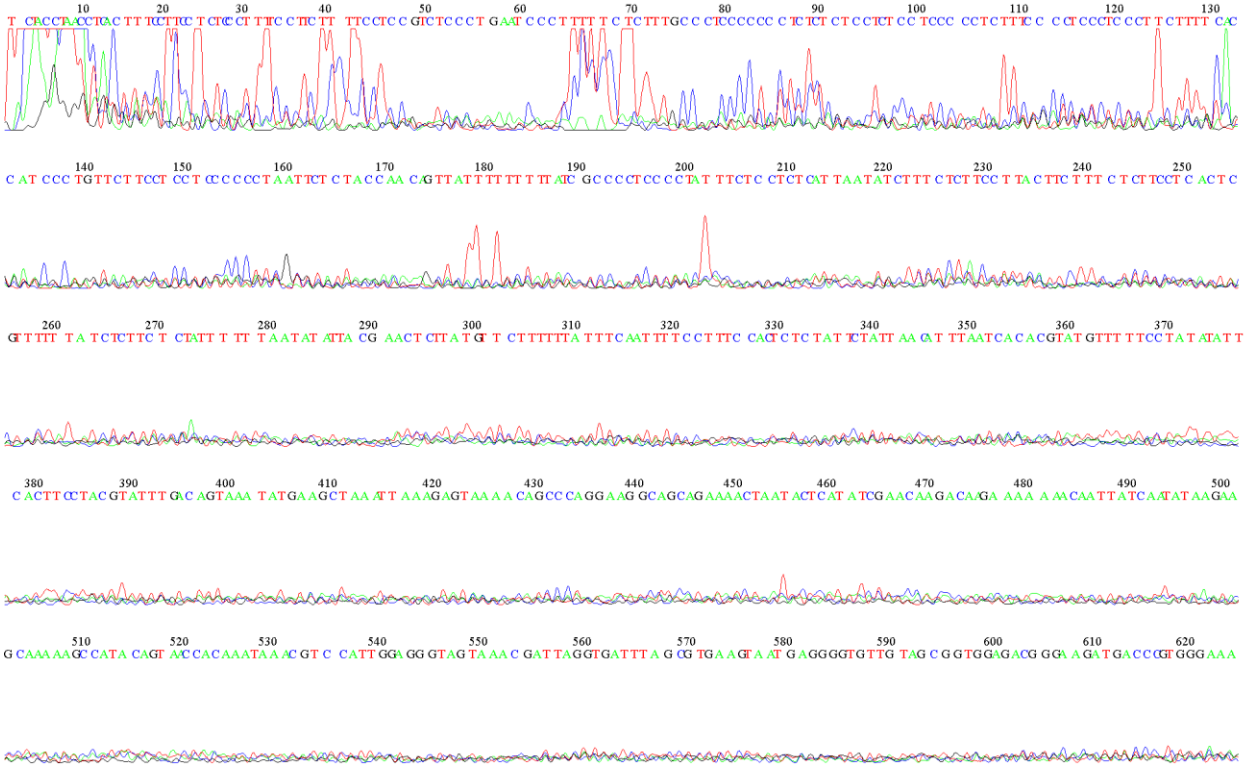

Figure 105: S20 – nrITS – Bad quality sequence – AHPA-BC071

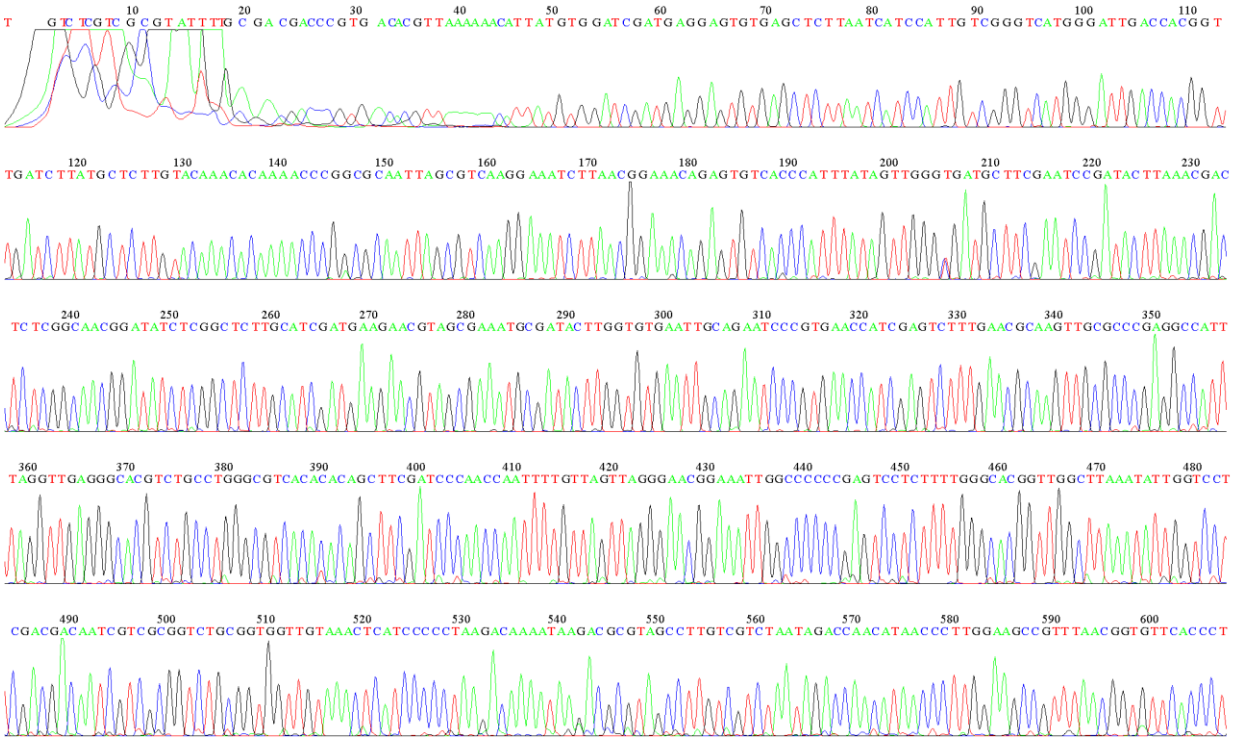

Figure 106: S22 – nrITS – *Actaea racemosa* – AHPA-BC053

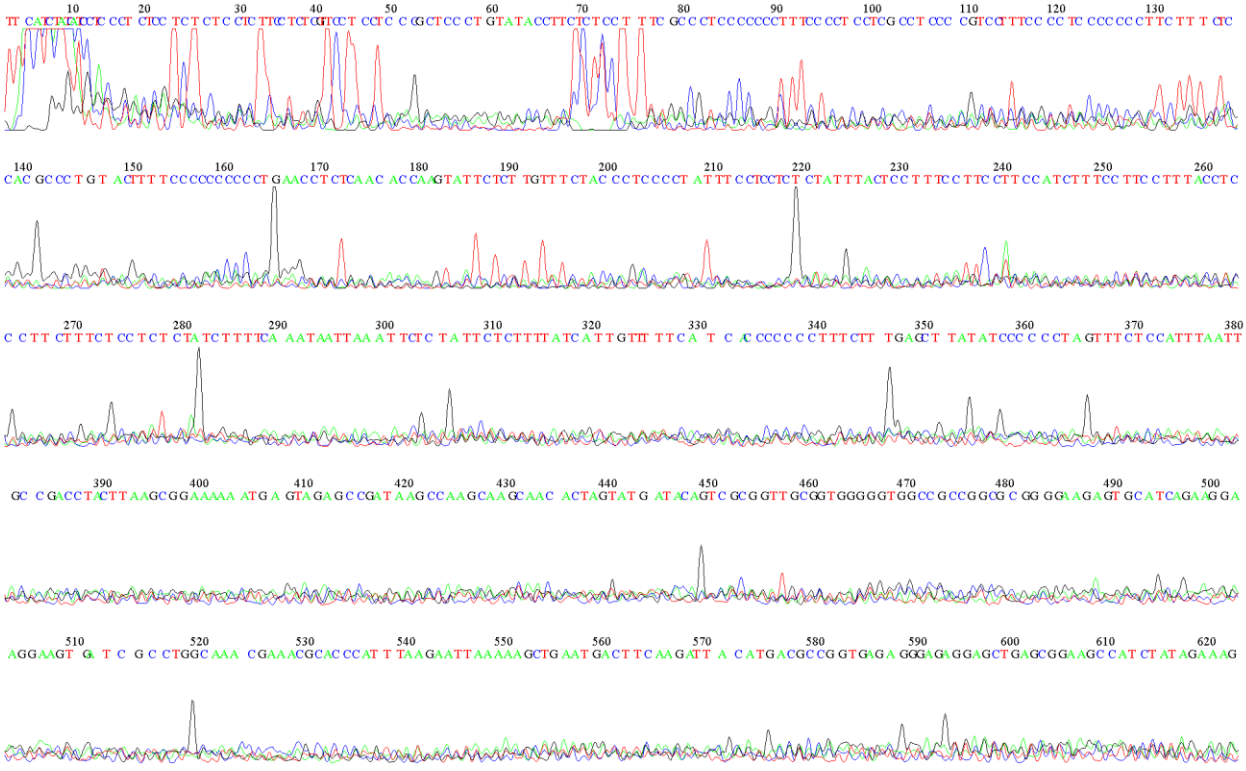

Figure 107: S24 – nrITS – Bad quality sequence – AHPA-BC057

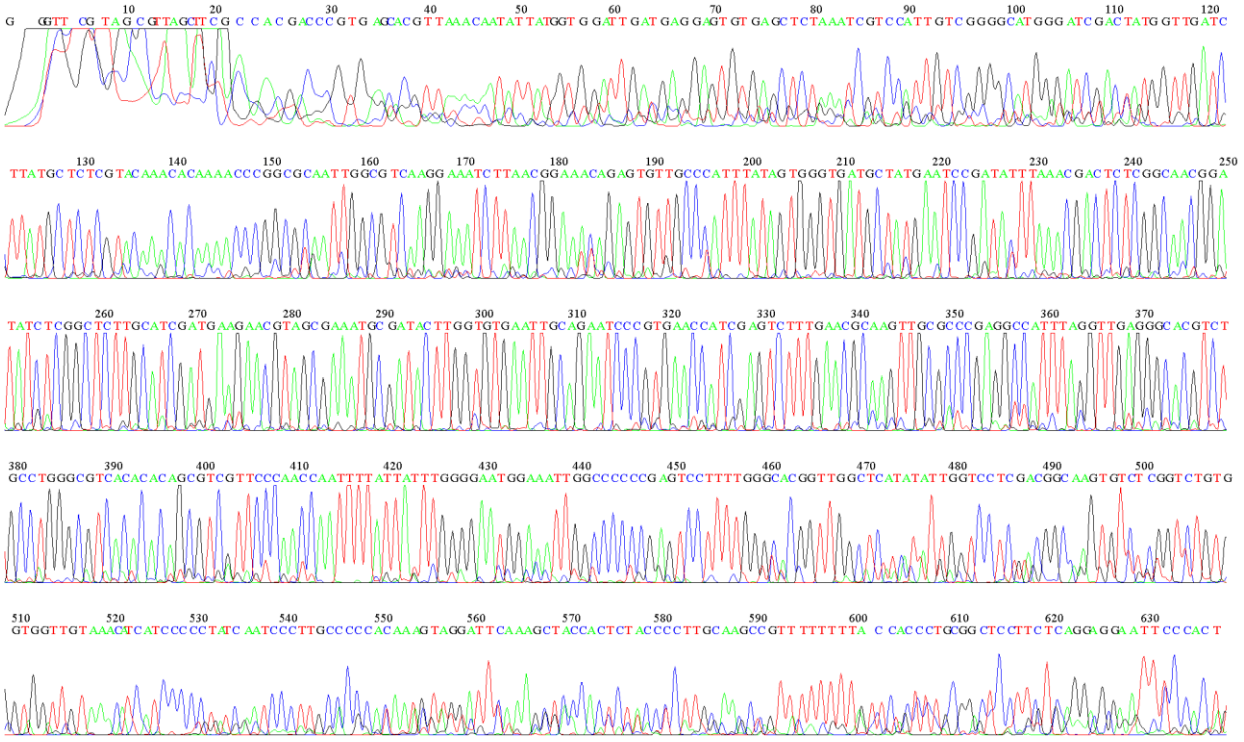

Figure 108: S25 – nrITS – *Actaea dahurica* – AHPA-BC058

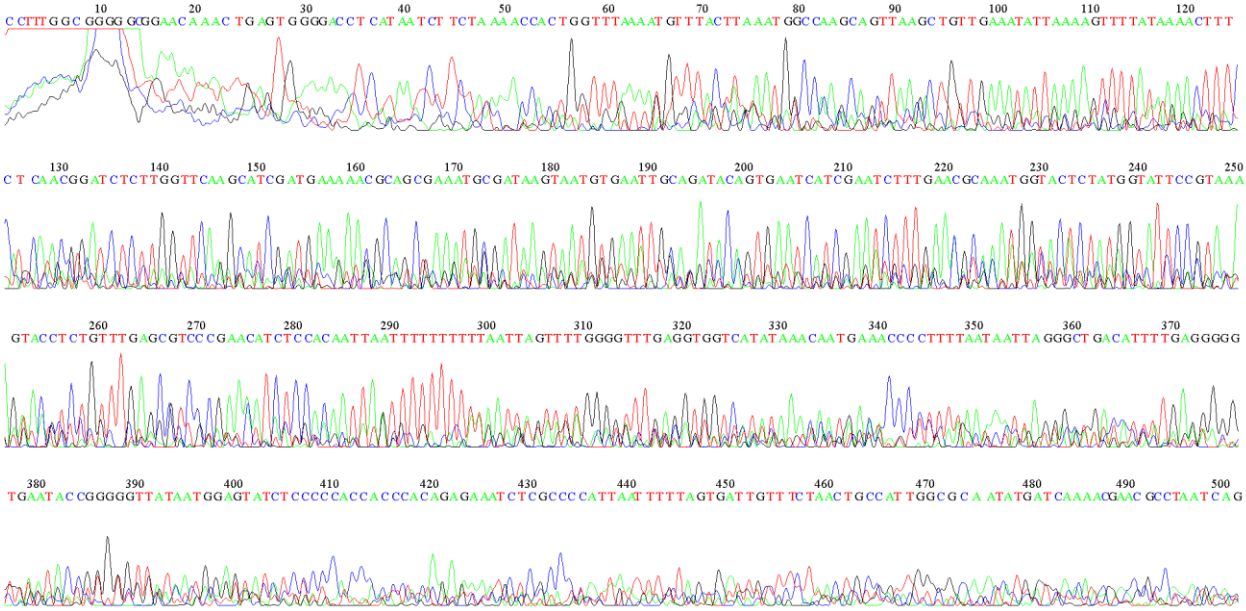

Figure 109: S28 - nrITS – Bad quality sequence– AHPA-BC063

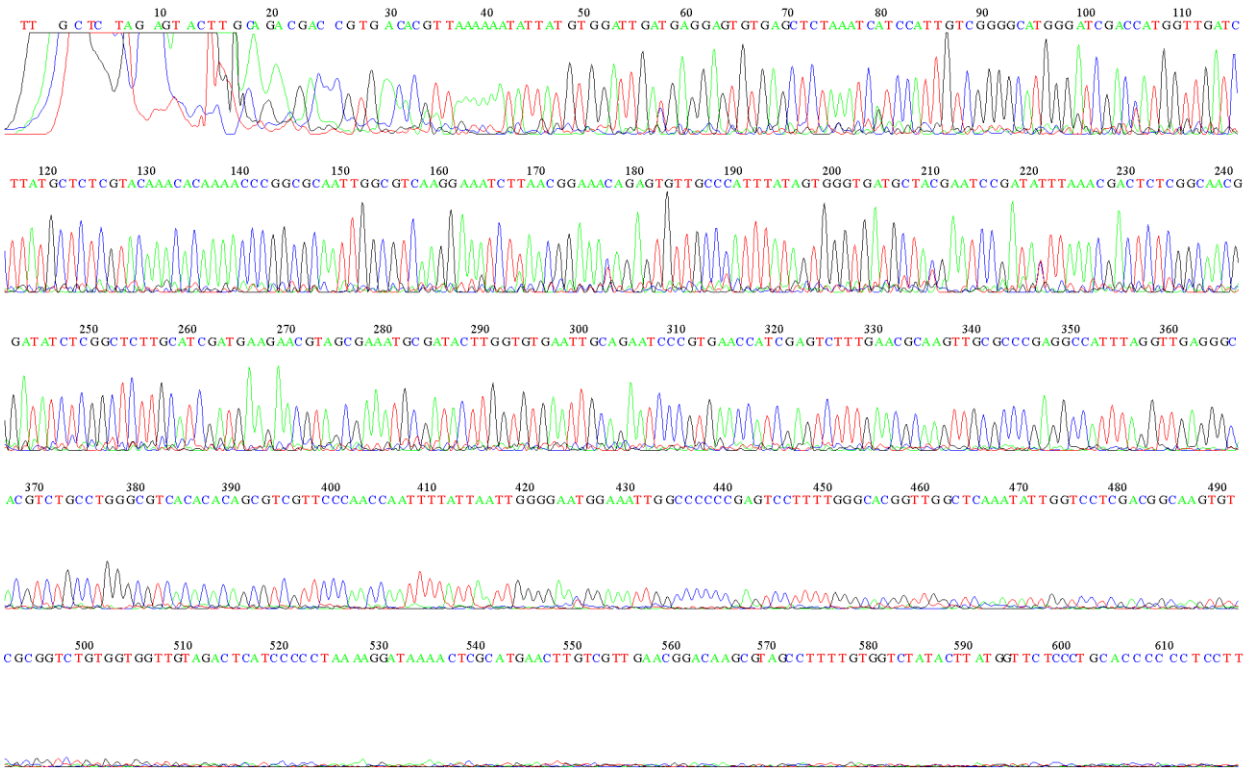

Figure 110: 1032 – nrITS – *Actaea dahurica* – AHPA-BC001.

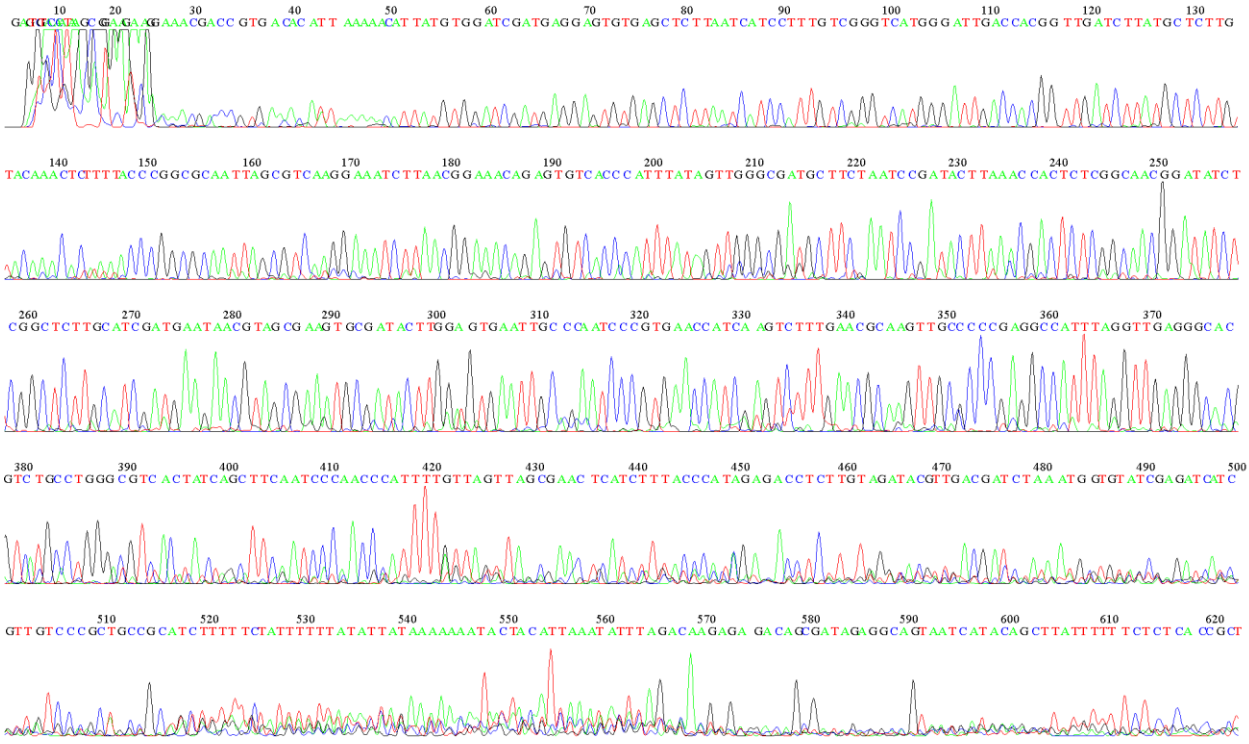

Figure 111: 1095 – nrITS – *Actaea racemosa* – AHPA-BC007

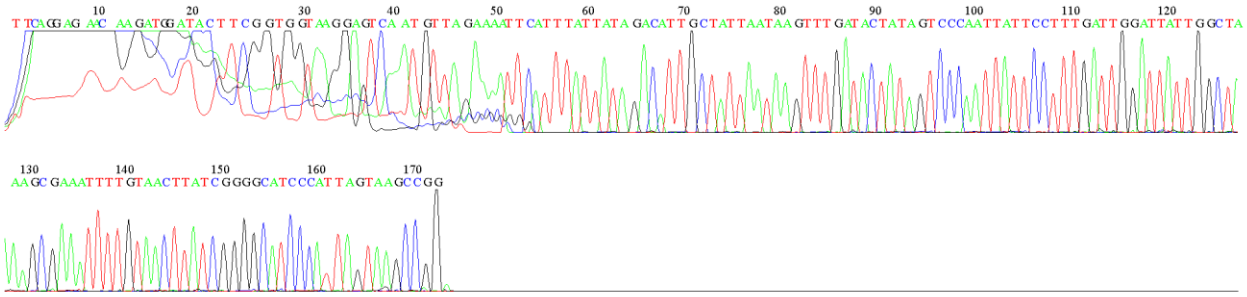

Figure 112: 1096 - *matK* – *Actaea racemosa* – AHPA-BC008

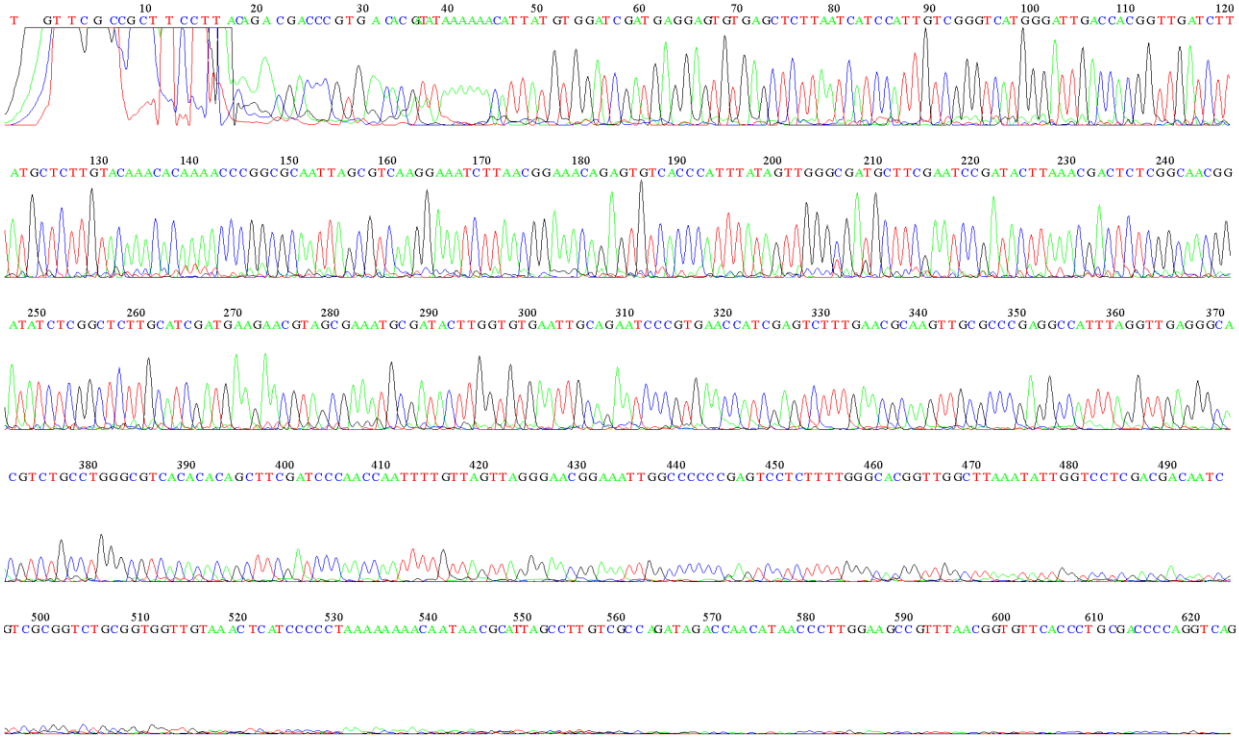

Figure 113: 1101 – nrITS - *Actaea racemosa* – AHPA-BC013

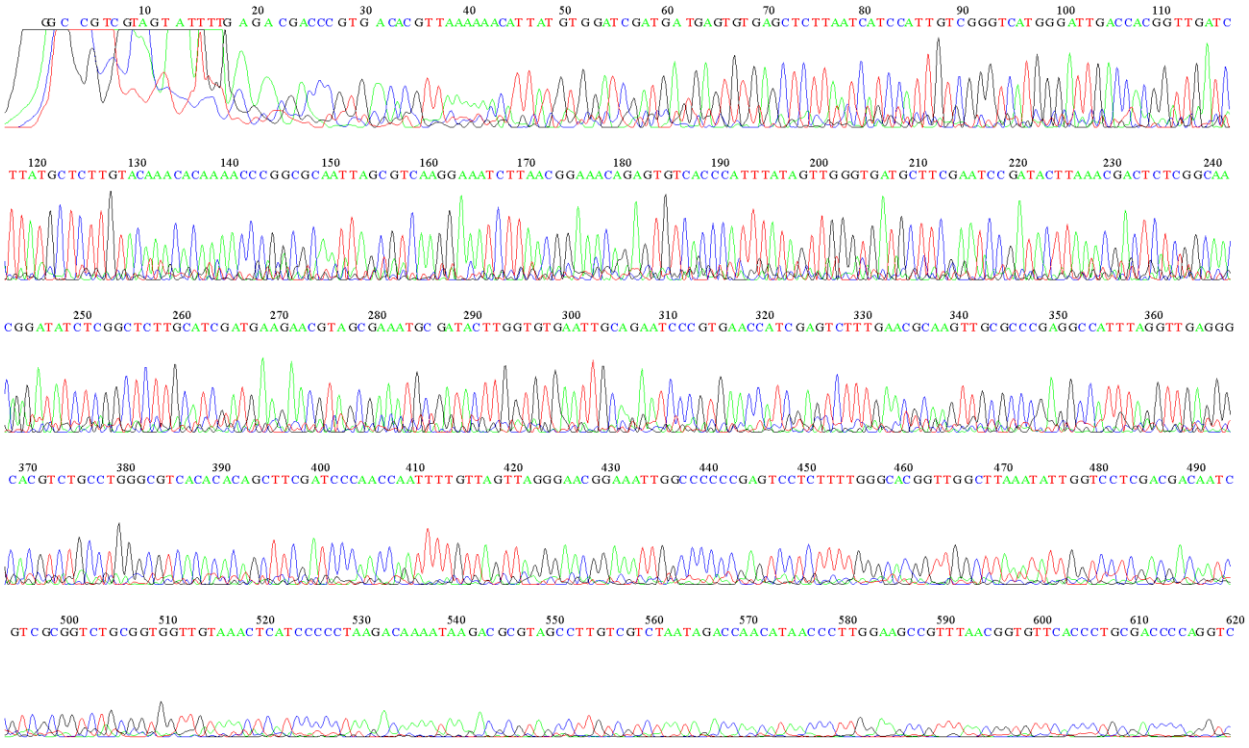

Figure 114: 1116 – nrITS – *Actaea racemosa* – AHPA-BC039

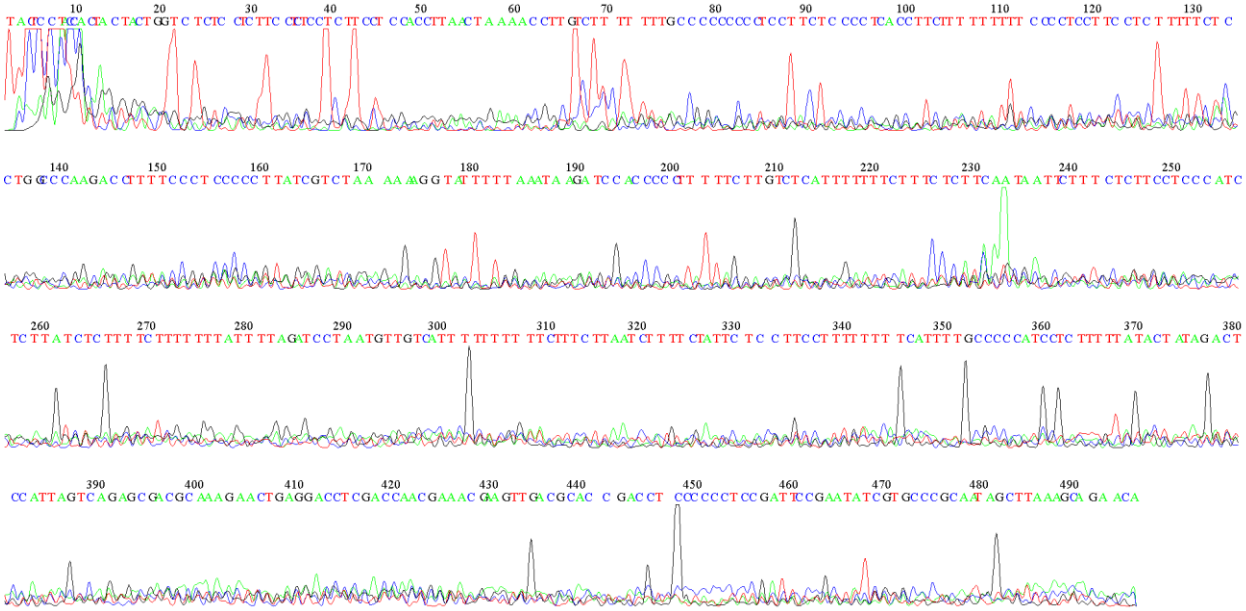

Figure 115: 1115 – nrITS – Bad quality sequence – AHPA-BC038

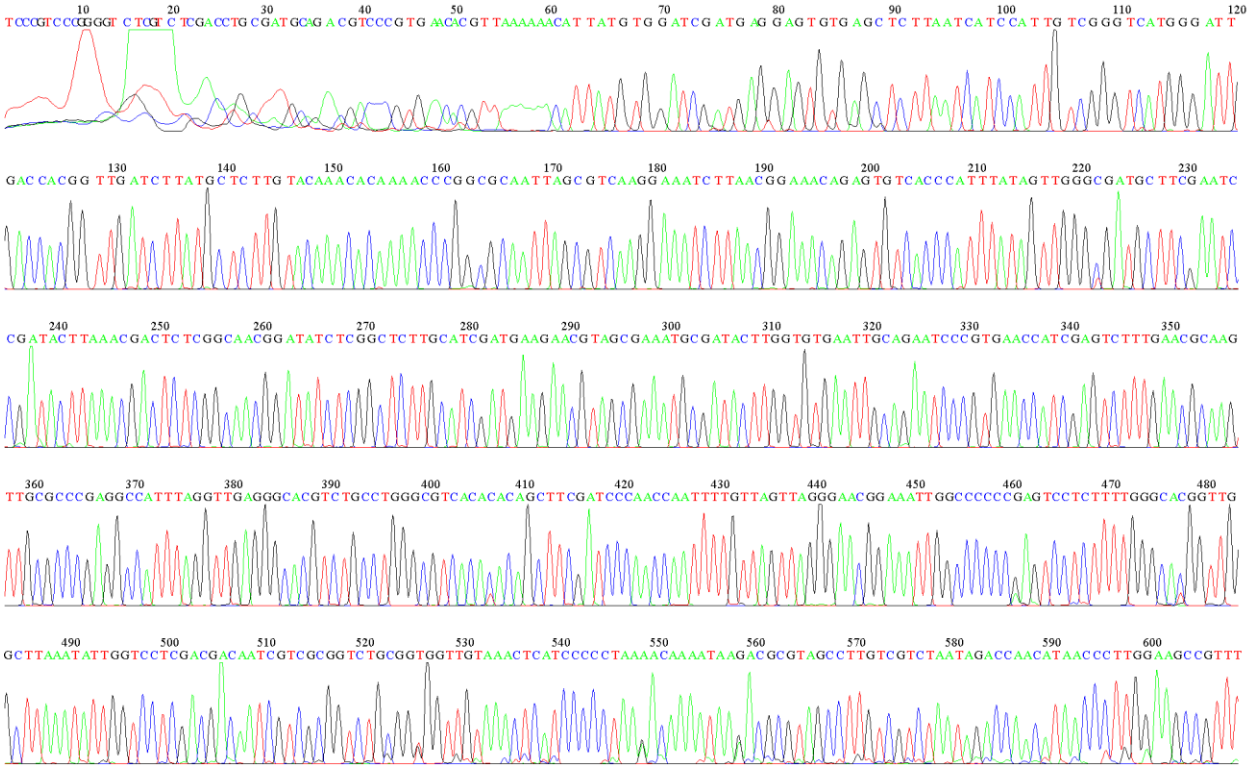

Figure 116: 1117 – nrITS – *Actaea racemosa* – AHPA-BC040

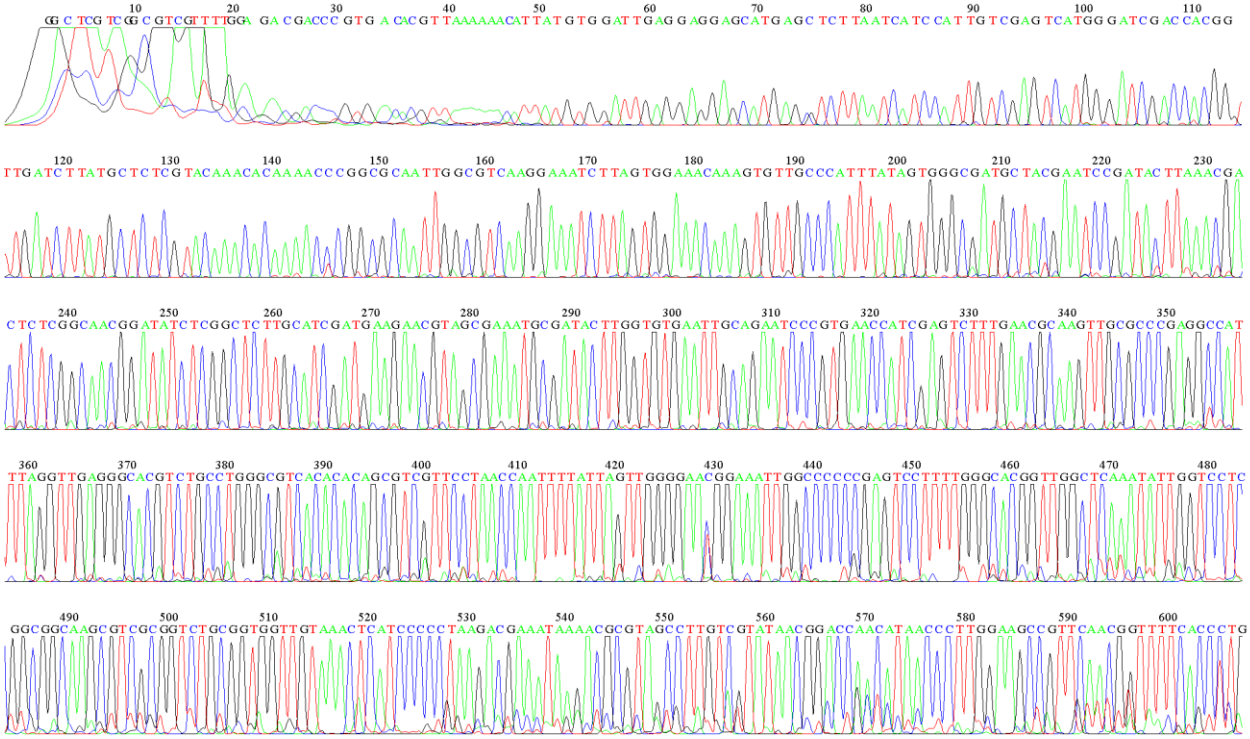

Figure 117: 1119 – nrITS – *Actaea cimicifuga* – AHPA-BC044

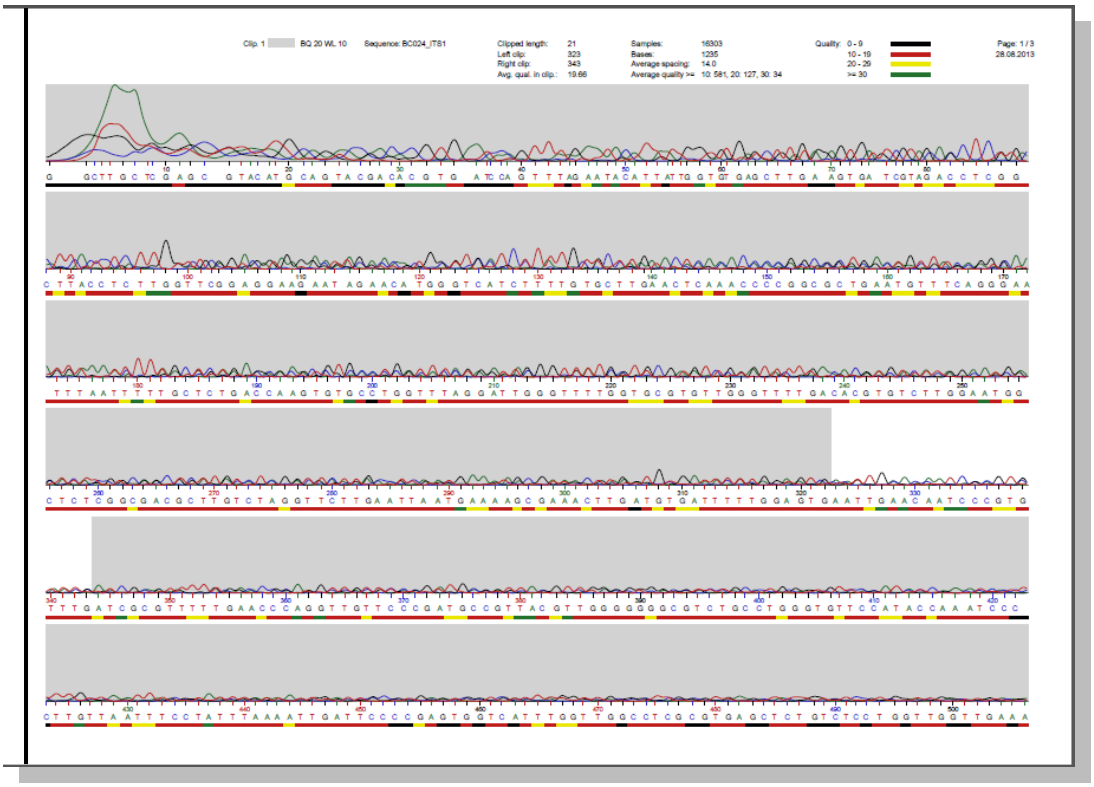

Figure 118: BC024 – nrITS –*Bad Quality Sequence* - product 104

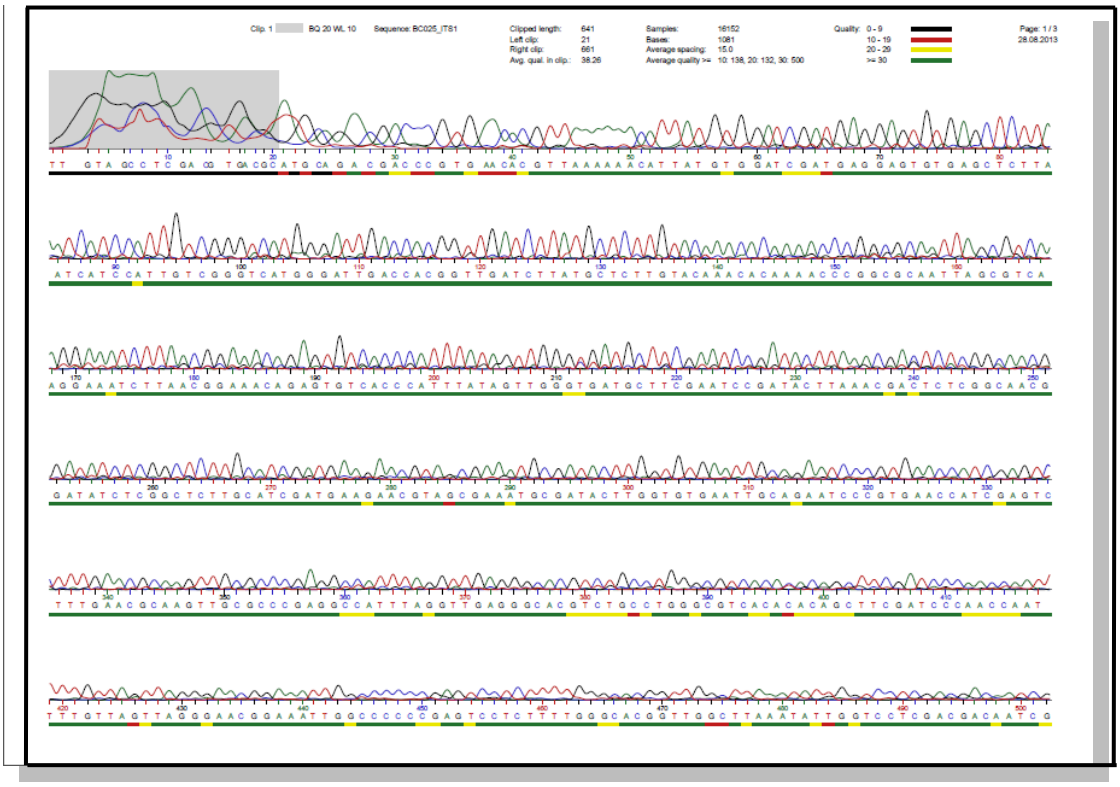

Figure 119: BC025 – nrITS – *Actaea racemosa* – Product 105

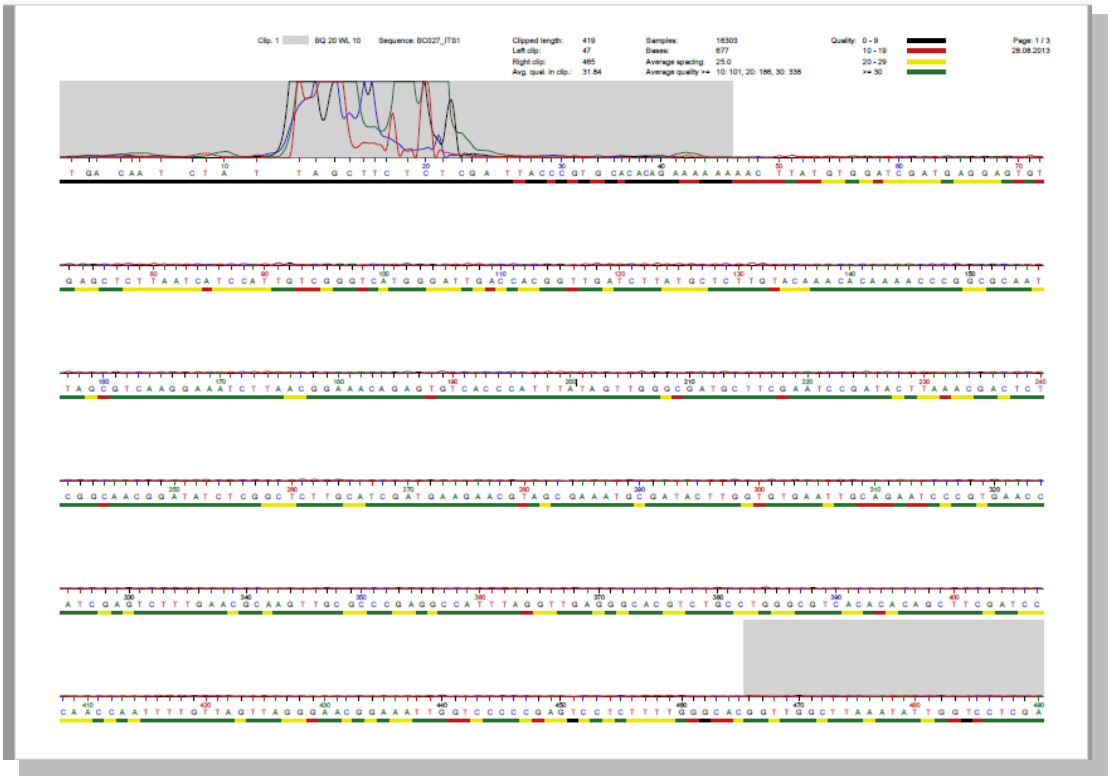

Figure 120: BC027 – nrITS – *Actaea racemosa* – Product 107

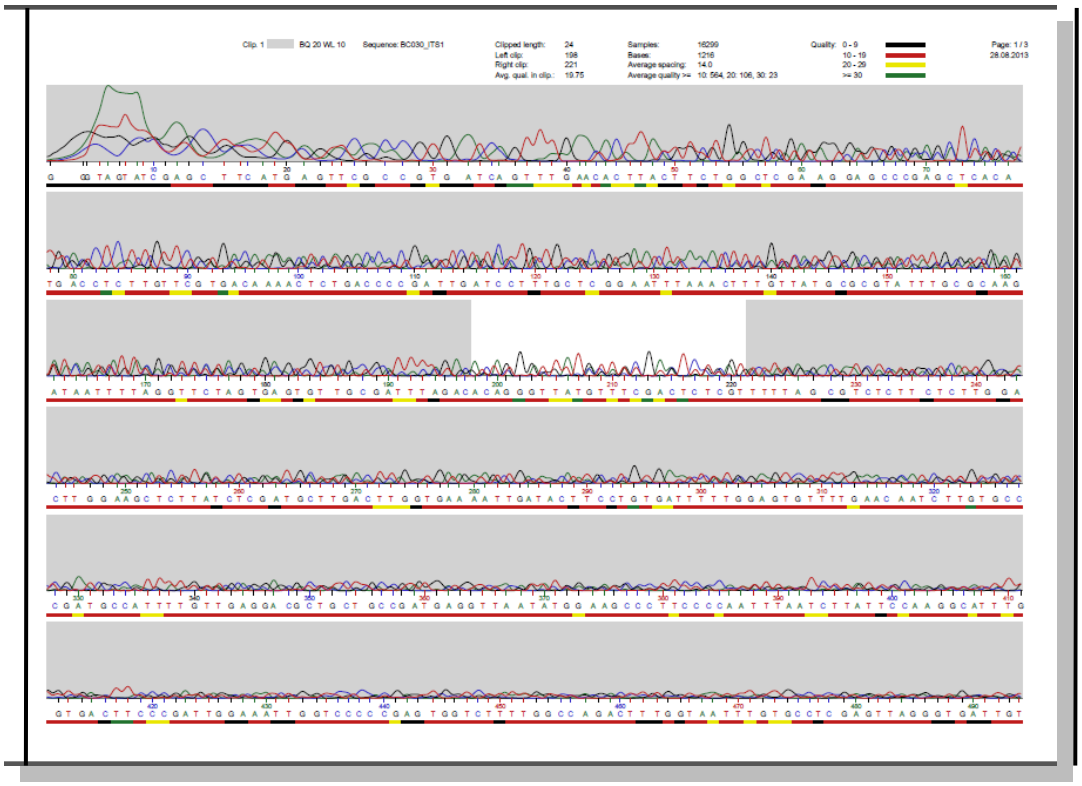

Figure 121: BC030 – nrITS – Bad quality sequence – Product 120

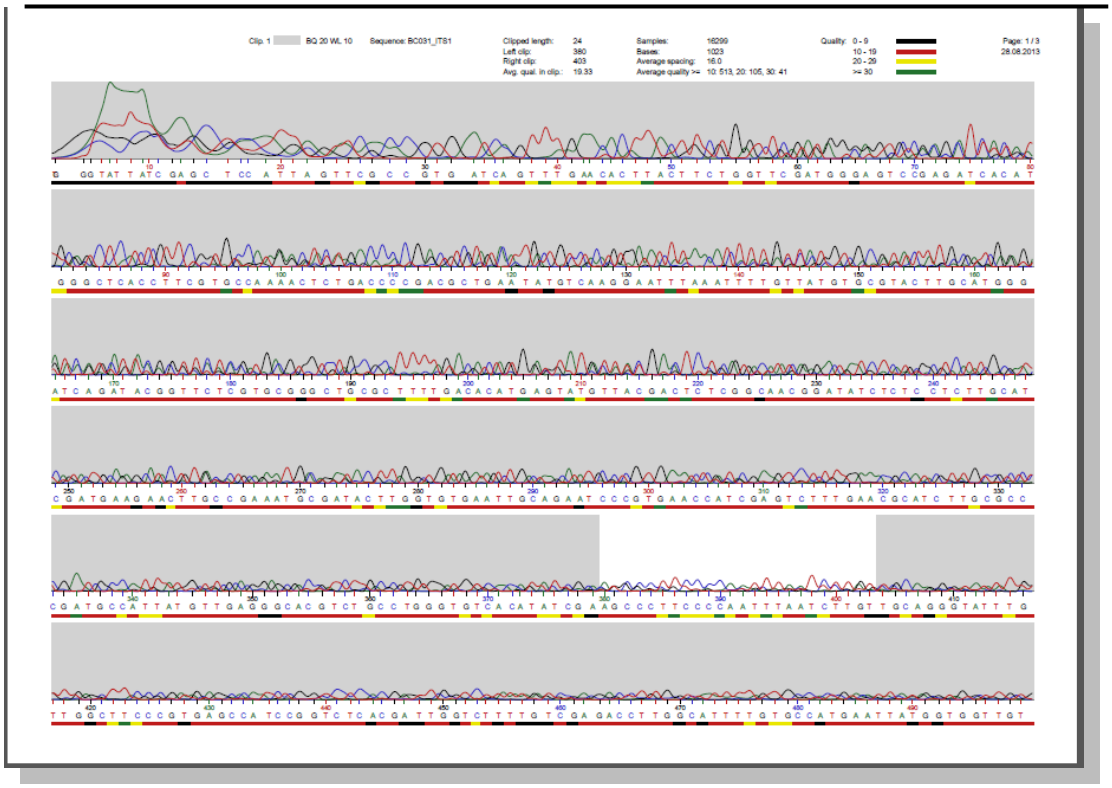

Figure 122: BC031 – nrITS – Bad quality sequence – Product 121

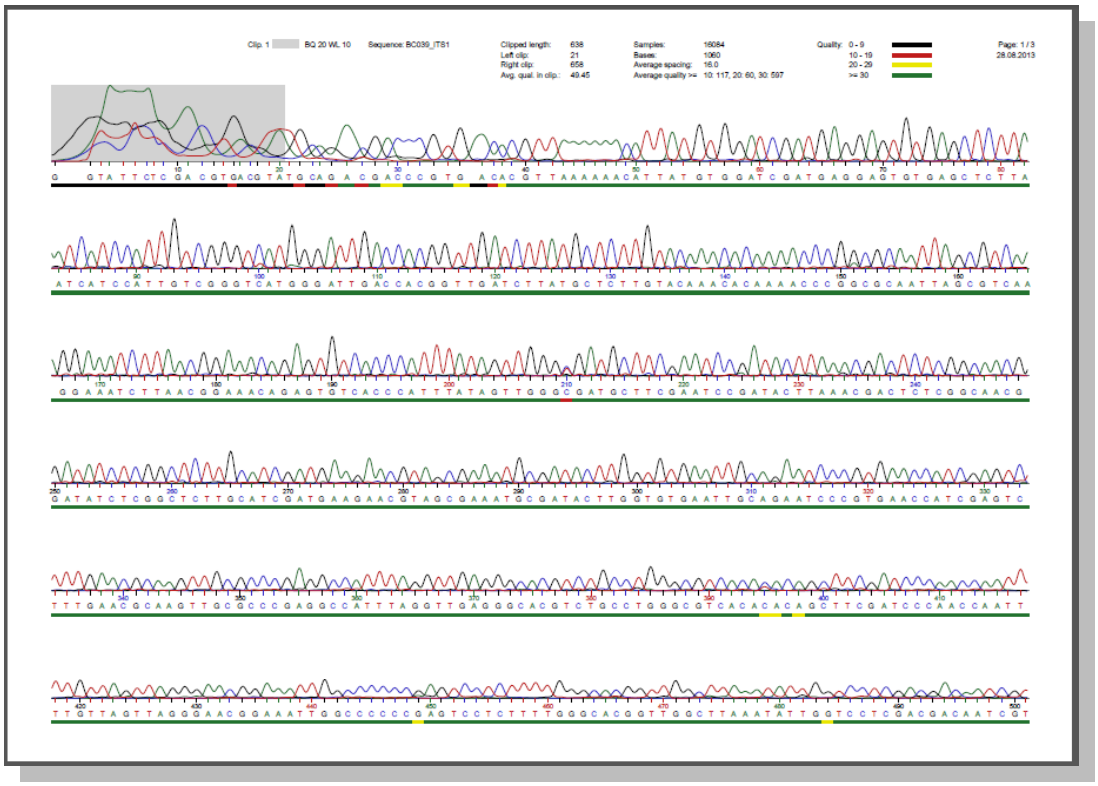

Figure 123: BC039 – nrITS – *Actaea racemosa* – product 081

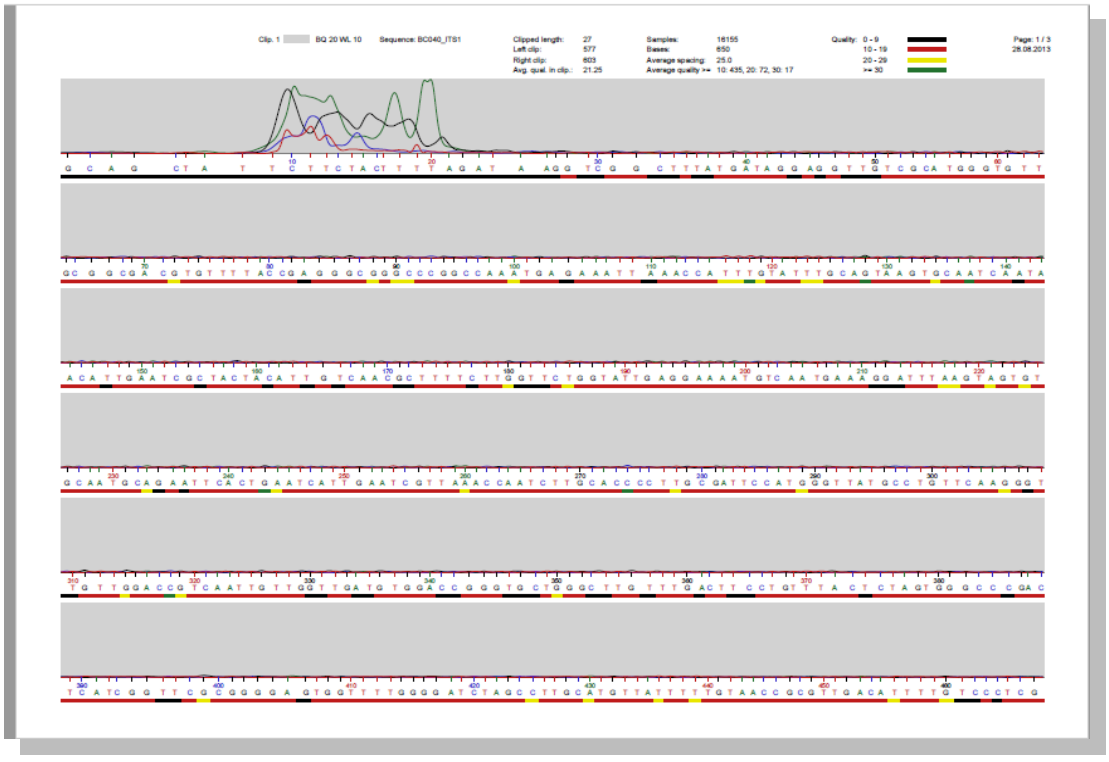

Figure 124: BC040 – nrITS – *Actaea racemosa* – Product 082

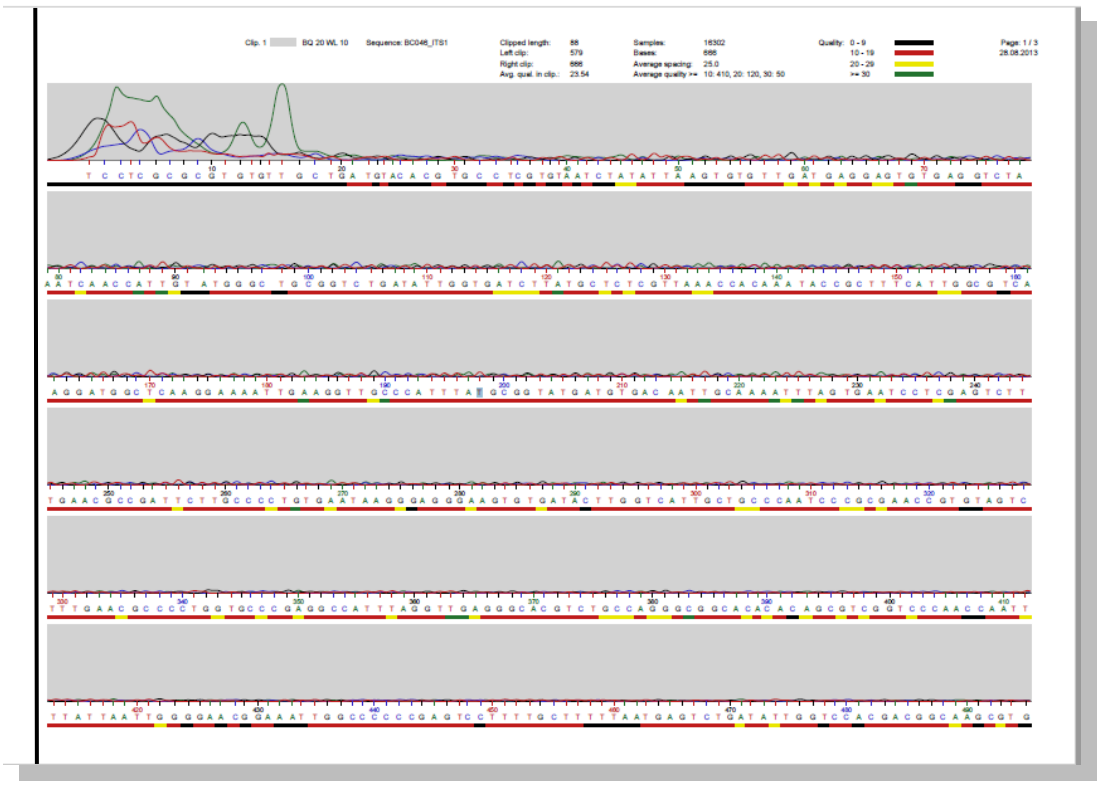

Figure 125: BC046 – nrITS – SynoPhyto - Ch.B.13884K116-A

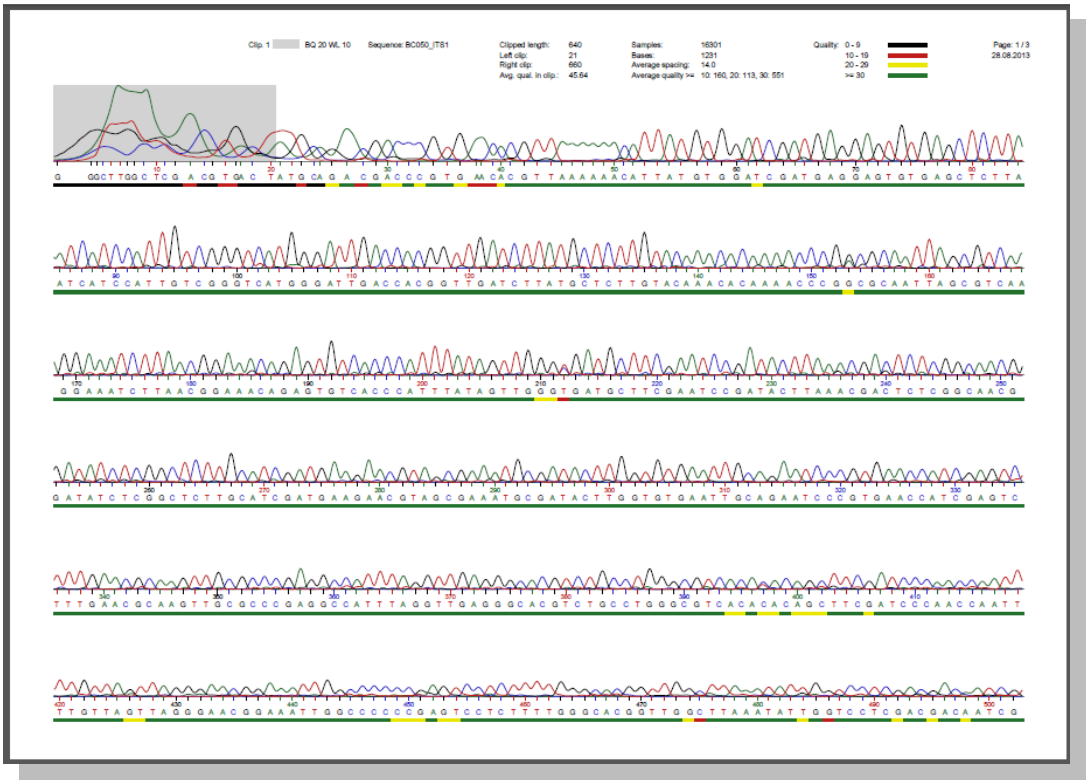

Figure 126: BC050 – nrITS – *Actaea racemosa* - DR10-014-A

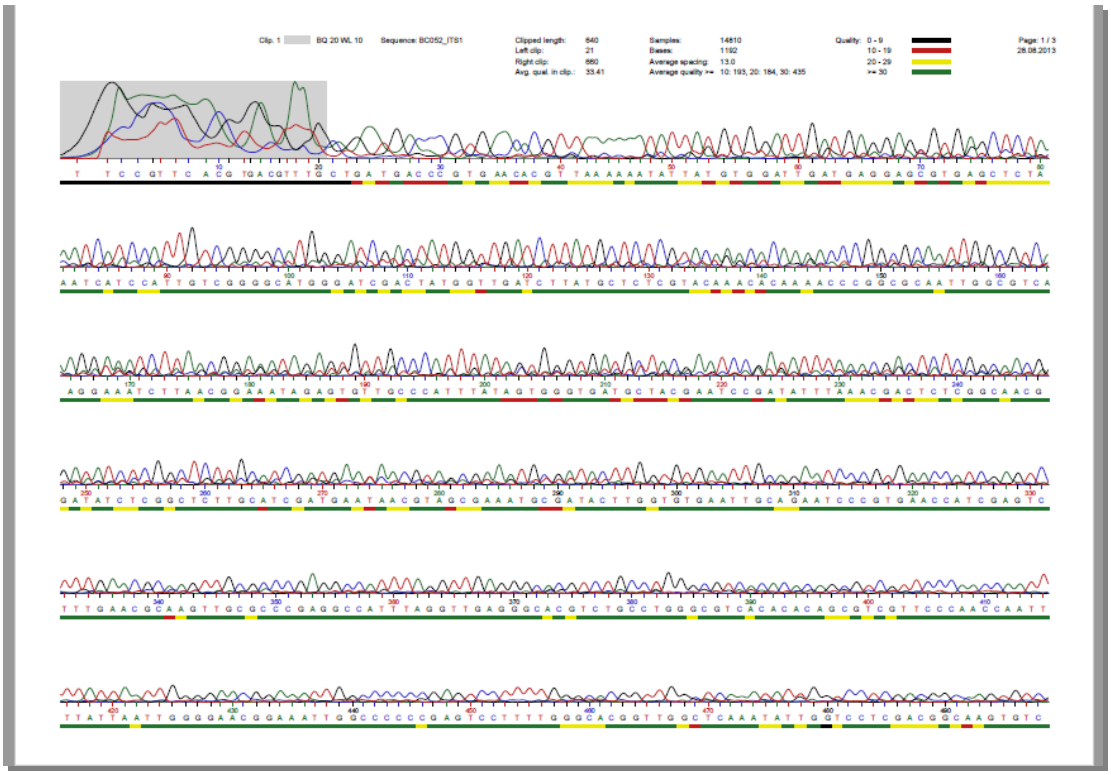

Figure 127: BC052 – nrITS – *Actaea dahurica* – CAMAG 58448

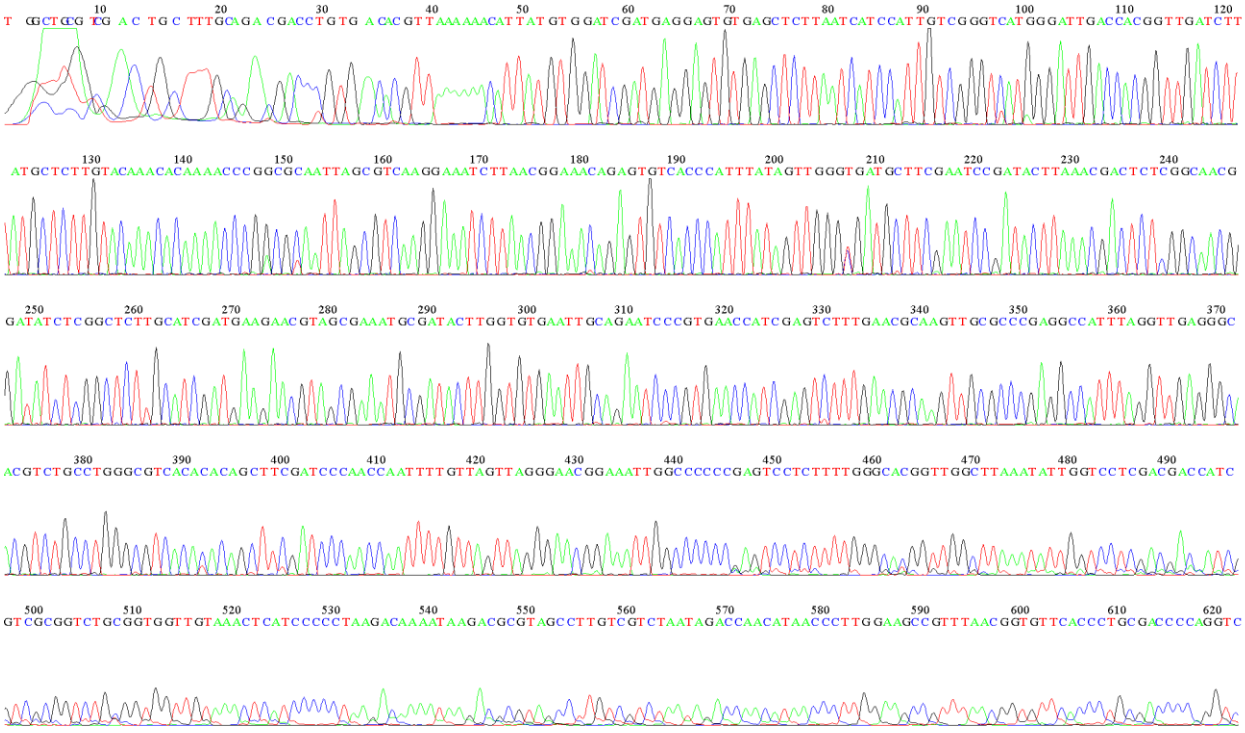

Figure 128: BC055 – nrITS – *Actaea racemosa* – Staffort, DHU.

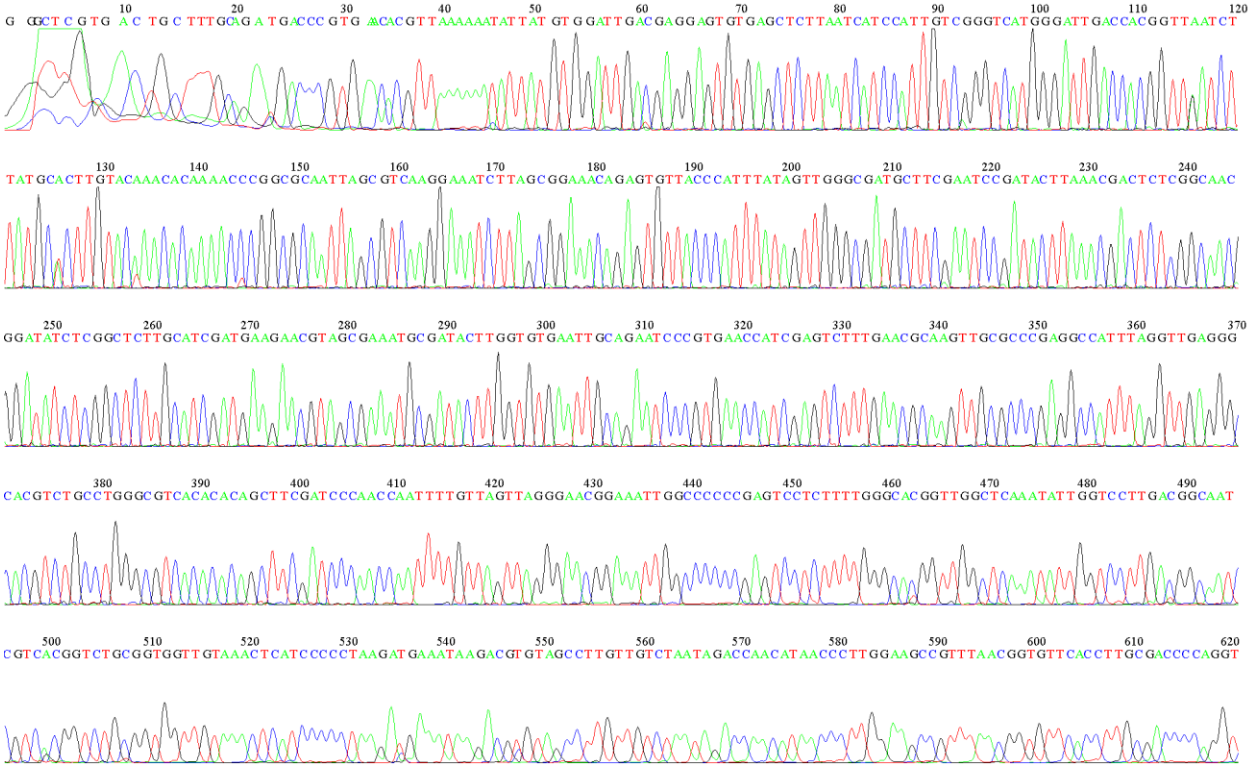

Figure 129: BC056 – nrITS – *Actaea spicata* – Staffort, DHU

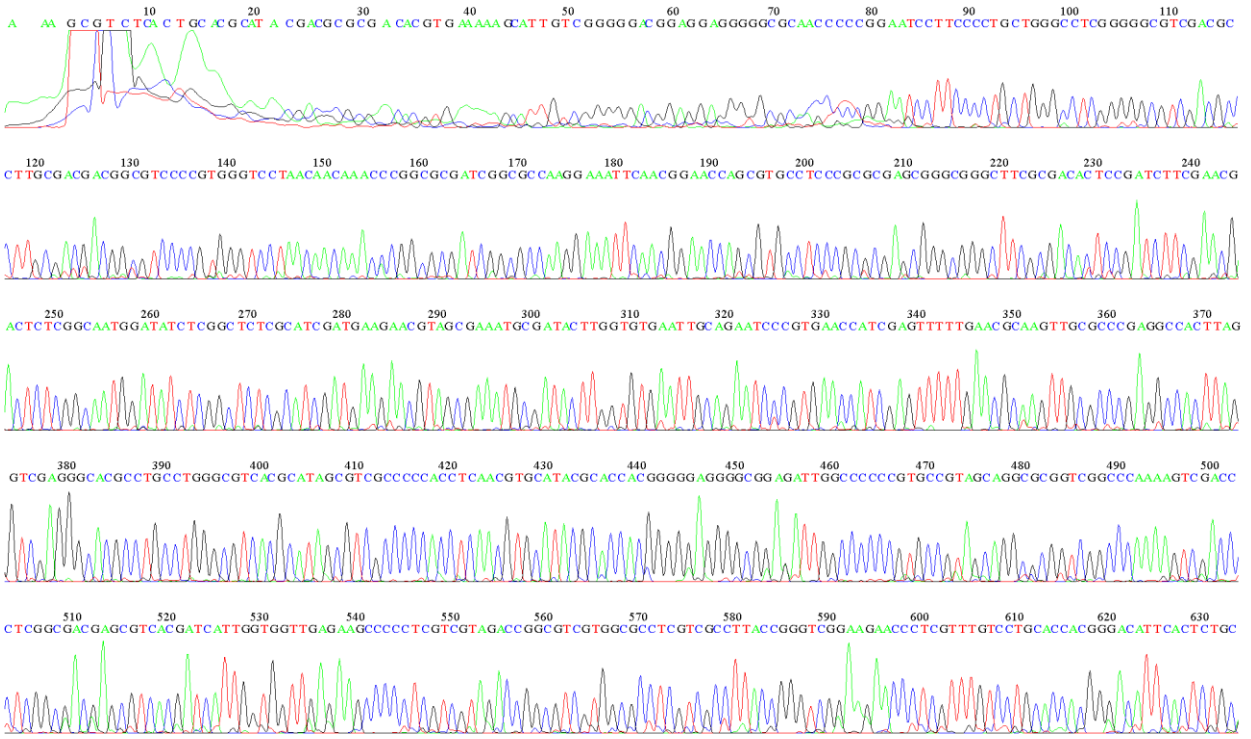

Figure 130: BC057 – nrITS – *Caulophyllum thalictroides* – Staffort, DHU

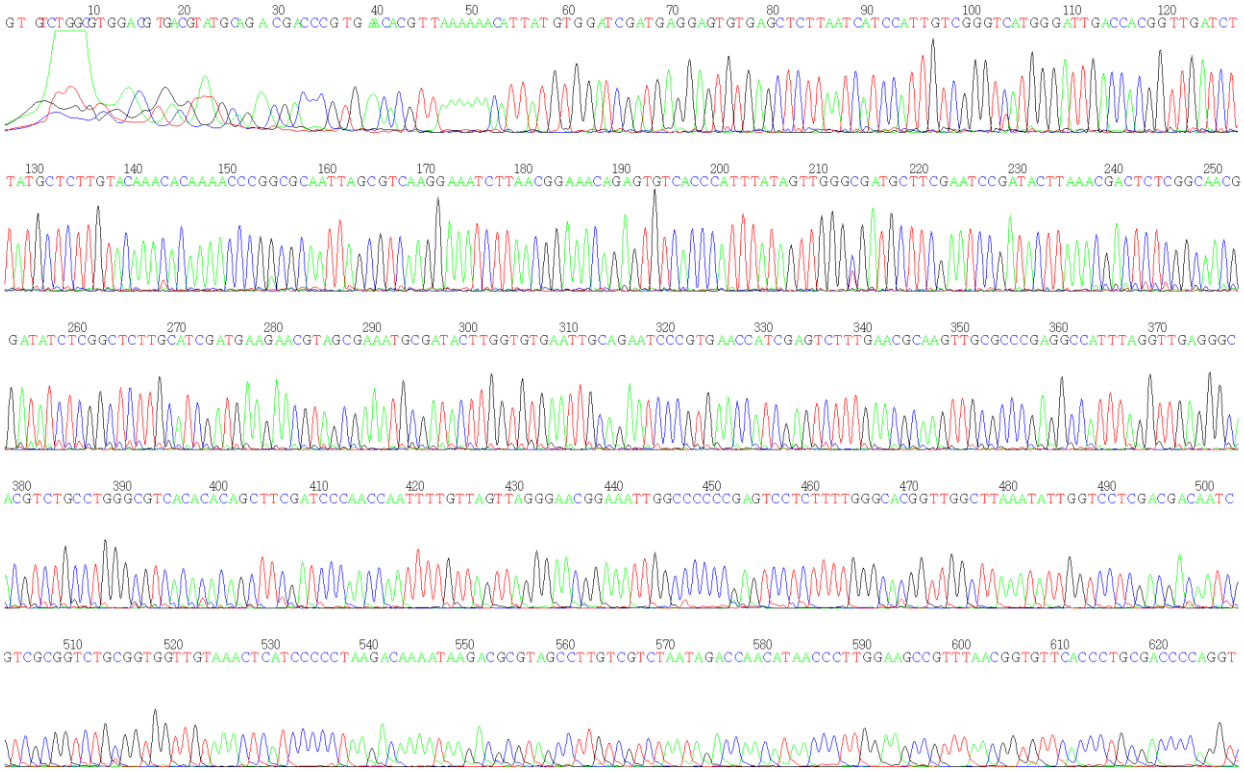

Figure 131: 157 – nrITS – *Actaea racemosa* – CAMAG - 52784BRM

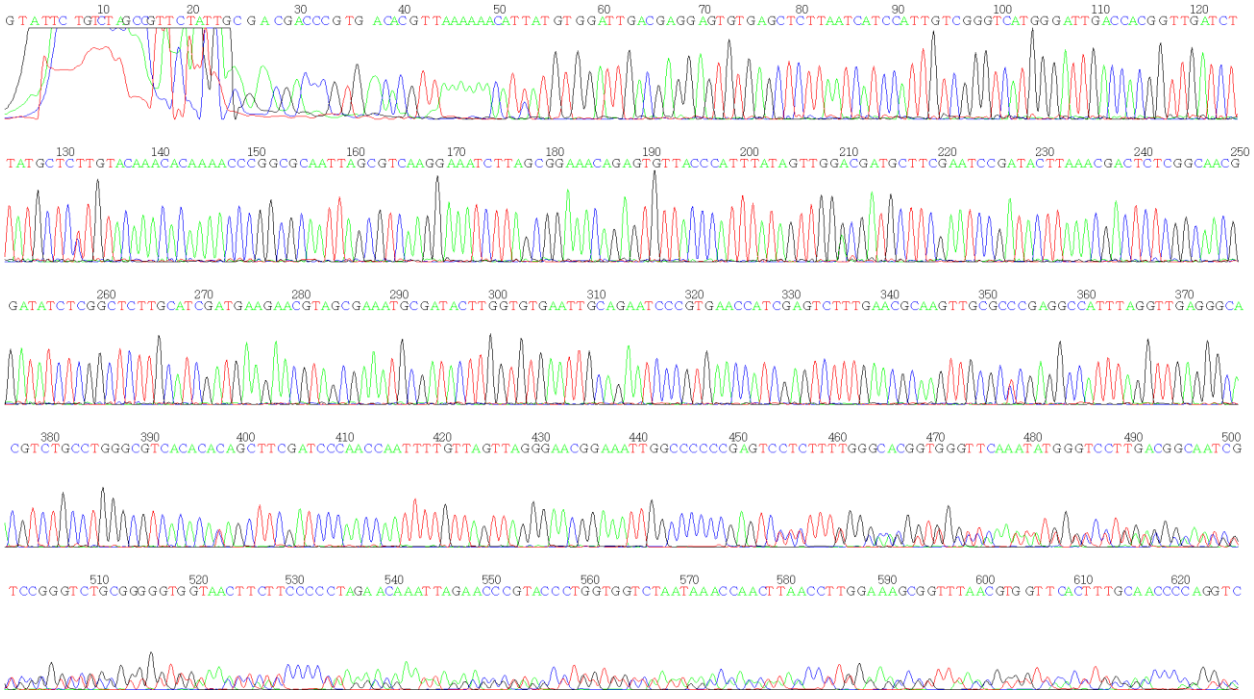

Figure 132: 158 – nrITS – *Actaea pachypoda* – CAMAG - 52483BRM

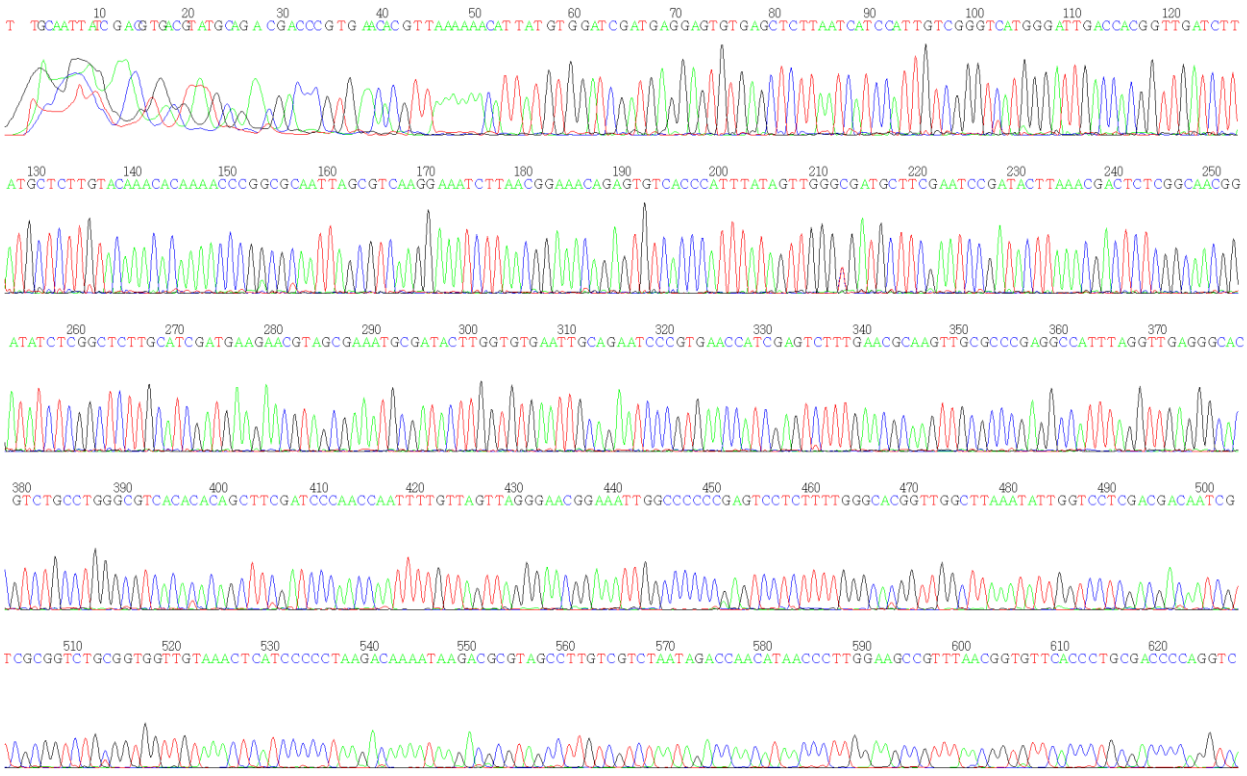

Figure 133: 161 – nrITS – *Actaea racemosa* – CAMAG - 52789BRM

8.5 qPCR data for hepatotoxicity arrays from *Actaea* treated HepaRG™ cells

8.5.1 Experiment 1 – Pilot study

8.5.1.1 Full C_t data for the pilot gene expression study

Well	1 Normal	2 APAP	3 DMSO	4 CD	5 CF	6 CR
A01	24.71	23.94	24.71	26.56	25.21	25.89
A02	29.41	32.77	29.41	31.16	30.73	30.99
A03	25.65	28.60	25.65	26.95	26.56	26.81
A04	22.67	26.22	22.67	23.53	22.69	23.05
A05	22.73	24.56	22.73	22.97	23.11	23.21
A06	19.93	21.19	19.93	20.02	19.59	19.79
A07	22.70	23.92	22.7	22.96	22.68	23
A08	23.70	23.86	23.7	24.2	23.88	24.19
A09	26.42	25.71	26.42	27.72	26.75	27.35
A10	28.85	29.91	28.85	28.73	28.35	29.31
A11	32.62	33.40	32.62	33.23	32.69	32.32
A12	28.11	29.94	28.11	27.75	27.26	27.74
B01	31.13	31.63	31.13	32.32	31.69	31.96
B02	25.32	25.70	25.32	25.76	25.16	25.33

B03	22.56	23.81	22.56	22.71	22.6	22.88
B04	30.76	32.03	30.76	33.12	31.3	30.79
B05	25.14	26.84	25.14	24.76	24.1	24.62
B06	26.08	27.40	26.08	26.97	26.58	26.81
B07	22.19	22.22	22.19	22.25	21.71	21.92
B08	29.69	28.88	29.69	30.34	29.67	29.94
B09	24.64	25.13	24.64	25.81	25.32	25.59
B10	37.43	35	35	35	35	35
B11	30.88	31.16	30.88	33.65	30.97	28.53
B12	33.63	38.19	33.63	34.61	35	35
C01	25.07	26.36	25.07	24.96	24.19	24.79
C02	24.63	24.73	24.63	24.67	24.66	24.82
C03	23.39	23.83	23.39	23.41	23.63	23.67
C04	20.70	25.60	20.7	23.57	25.26	24.01
C05	23.26	23.54	23.26	23.86	23.35	23.47
C06	26.63	26.44	26.63	26.75	26.71	26.85
C07	21.95	22.40	21.95	23.1	22.91	22.86
C08	31.58	34.42	31.58	33.14	35	32.66
C09	25.87	25.85	25.87	25.76	25.75	25.68
C10	25.70	23.48	25.7	25.64	25.23	25.5
C11	22.90	22.66	22.9	23.37	23.43	23.56
C12	26.04	26.50	26.04	26.51	26.25	26.09
D01	23.82	30.79	23.82	27.05	27.96	26.67
D02	24.62	23.68	24.62	25.01	24.64	24.89
D03	23.56	27.16	23.56	23.99	24.89	24.66
D04	23.56	22.58	23.56	23.33	23.49	23.52
D05	23.68	25.20	23.68	22.05	22.4	22.69
D06	33.42	37.60	33.42	33.61	34.81	33.25
D07	22.36	22.60	22.36	22.39	22.3	22.54
D08	26.44	26.37	26.44	26.22	25.76	26.04
D09	25.97	27.22	25.97	26.09	26.29	26.2
D10	21.69	23.16	21.69	21.34	21.14	21.52
D11	22.45	23.77	22.45	22.74	21.97	22.59
D12	26.86	27.54	26.86	27.57	26.88	27.06
E01	32.35	35	32.35	33.95	35	35
E02	24.76	25.02	24.76	24.35	24.42	24.52
E03	23.65	24.88	23.65	24.26	23.85	24.1
E04	23.73	26.16	23.73	25.01	24.63	24.66
E05	25.76	27.47	25.76	25.72	25.15	25.63
E06	29.51	33.17	29.51	30.76	30.65	29.97
E07	28.59	30.63	28.59	28.56	26.59	27.45
E08	23.89	24.41	23.89	24.96	25.1	24.77
E09	25.22	25.83	25.22	25.01	24.88	25.05
E10	27.05	29.33	27.05	27.24	26.68	27.32

E11	24.89	25.61	24.89	24.78	24.68	24.86
E12	24.18	25.27	24.18	23.42	23.68	23.81
F01	26.61	31.50	26.61	26.23	26	26.12
F02	35	35	35	35	32.15	32.6
F03	25.57	25.62	25.57	25.91	25.55	25.87
F04	24.60	25.07	24.6	25.11	24.87	25.24
F05	23.66	23.97	23.66	23.89	23.36	23.71
F06	22.26	24.59	22.26	22.59	21.98	22.34
F07	24.22	25.21	24.22	24.47	23.98	24.46
F08	23.28	23.92	23.28	23.7	23.03	23.44
F09	33.57	34.97	33.57	35	34.04	33.83
F10	25.79	29.26	25.79	25.23	27.15	25.91
F11	19.50	20.76	19.5	20.01	19.63	19.77
F12	18.26	20.96	18.26	17.1	17.87	17.73
G01	18.06	18.58	18.06	17.57	16.84	17.48
G02	26.85	27.92	26.85	27.48	27.12	27.76
G03	29.04	31.93	29.04	30.17	29.6	29.29
G04	35.24	37.80	35	34.45	35	34.49
G05	25.37	25.10	25.37	25.46	24.71	25.21
G06	25.49	27.77	25.49	26.15	25.96	26.07
G07	29.56	30.58	29.56	29.71	29.6	30.04
G08	27.76	29.66	27.76	31.39	32	31.29
G09	24.18	24.55	24.18	23.9	23.95	24.02
G10	20.84	20.25	20.84	21.67	21.04	21.18
G11	25.32	24.82	25.32	24.94	25.48	25.57
G12	26.85	26.39	26.85	26.96	26.83	26.95
H01	16.95	18.40	16.95	17.02	16.6	16.94
H02	19.37	20.37	19.37	19.31	19.65	19.59
H03	18.58	20.98	18.58	18.43	18.12	18.13
H04	25.78	25.97	25.78	25.37	24.8	25.31
H05	18.69	18.93	18.69	18.25	18.08	18.25
H06	35	35	35	35	35	35
H07	23.27	23.59	23.27	23.82	23.11	23.34
H08	23.39	23.48	23.39	23.78	23.08	23.38
H09	23.34	23.47	23.34	23.76	23.04	23.19
H10	18.38	18.47	18.38	18.22	18.45	18.41
H11	18.49	18.56	18.49	18.57	18.46	18.57
H12	18.03	18.23	18.03	18.1	18.37	18.29

8.5.2 Experiment 2 – Triplicate arrays

8.5.2.1 Full C_t data for the gene expression study for Black Cohosh and Sheng Ma extract treatment of HepaRG™ cells.

Well	1 Normal	2 APAP	3 DMSO	4 DMSO	5 DMSO	6 CD 100	7 CD 100	8 CD 100	9 CD 20	10 CD 20	11 CD 20	12 CD 5	13 CD 5	14 CD 5	15 CR 100	16 CR 100	17 CR 100	18 CR 20	19 CR 20	20 CR 20	21 CR 5	22 CR 5	23 CR 5
A01	26.96	24	25.07	25.07	25.29	25.42	25.75	25.18	25.55	25.03	25.4	25.63	25.57	25.72	26.99	26.03	26.01	25.65	26.67	25.37	25.12	25.18	25.39
A02	30.61	33.43	29.12	29.18	29.64	29.14	29.5	28.97	29.58	29.63	29.27	29.87	30.02	30.14	31.87	30.08	30.15	30.14	30.7	30.05	29.23	29.34	29.59
A03	27.04	28.36	25.88	25.85	26.12	25.78	26.13	25.59	26.21	25.46	25.84	26.15	25.86	26.16	28.38	26.3	26.18	26.19	26.85	26.33	25.89	26.14	26.27
A04	24.01	26.55	23.68	23.65	23.9	23.49	23.73	23.61	23.75	23.23	23.72	23.89	24	24.06	25.51	24.52	24.13	24.11	25.09	23.83	23.72	23.77	24.27
A05	23.27	24.26	23.18	23.06	23.07	22.86	23.18	23.11	23.41	22.83	23.21	23.34	23.58	23.22	24.88	23.57	23.63	23.63	24.37	23.04	22.79	22.86	23.11
A06	19.88	21.15	20.02	19.95	19.74	19.71	20.05	19.74	20.25	19.66	20.31	20.23	20.35	20.13	21.8	20.29	20.43	20.54	21.12	20.06	19.79	19.85	20.17
A07	23.4	23.59	22.59	22.6	22.8	22.57	23.08	22.57	22.92	22.12	22.8	22.84	22.82	22.84	24.38	23.14	22.92	22.97	24.03	23.3	22.59	22.67	23.31
A08	24.64	23.6	23.79	23.53	23.82	23.67	24.02	23.67	23.9	23.48	23.9	24.08	24.02	24.05	25.59	24.41	24.33	24.24	24.99	24.26	23.87	24.04	24.56
A09	28.78	26.59	27.65	27.63	27.71	27.77	28.09	27.87	28.12	27.7	27.91	27.83	27.82	27.96	29.12	28.62	28.29	28.52	28.92	27.88	27.5	27.81	27.98
A10	29.64	29.88	27.76	27.66	28.95	28.36	28.97	28.36	28.35	27.64	28.22	28.2	28.05	28.37	31.08	29.59	28.2	28.19	29.75	29.95	28.68	28.3	30.06
A11	31.7	30.6	30.57	30.69	30.95	31.33	32.59	31.1	30.39	30.67	30.63	30.79	31.31	31.34	32.82	31.81	30.71	31.22	31.85	30.88	31.23	30.86	31.16
A12	27.77	29.09	27.75	27.85	27.23	27.33	27.8	27.57	27.69	27.35	27.93	27.98	27.83	27.71	28.89	28.26	28.29	28.12	28.98	27.26	27.15	27.52	28.13
B01	30.85	30.92	30.41	30.21	30.61	30.8	31.38	31.04	30.23	30.25	30.32	30.46	29.85	30.34	33.51	30.84	30.4	30.73	31.6	30.83	30.25	30.43	31.07
B02	26.35	25.88	25.96	25.86	26.01	25.9	26.27	25.77	26.31	25.76	26.16	26.21	26.28	26.15	27.63	26.49	26.42	26.5	27.62	26.66	26.72	26.79	26.97
B03	22.54	23	22.86	22.74	22.9	22.85	23.41	22.84	23.38	22.49	23.14	23.24	23.1	23.4	24.67	23.74	23.3	23.4	24.22	23.33	23.16	23.2	23.74
B04	31.97	32.56	30.6	30.19	31.44	31.48	32.02	31.5	31.27	30.92	31.08	31.01	32.4	31.2	32.04	33.28	31.77	31.82	32.64	31.88	32.21	31.21	34.45
B05	25.35	26.54	25.66	25.79	25.79	25.05	25.73	25.29	25.69	24.76	25.77	25.67	25.59	25.65	28.21	25.96	25.42	25.86	26.76	26.19	25.46	25.18	26.26
B06	26.75	26.58	25.41	25.11	25.57	25.56	25.78	25.19	25.61	25.1	25.47	25.71	25.63	25.85	27.74	25.84	25.9	25.72	26.4	26.12	25.68	25.89	25.92
B07	22.15	21.74	21.73	21.62	21.74	21.74	22.11	21.5	22.01	21.35	21.87	21.87	21.85	21.95	23.35	22.14	22.22	22.07	22.83	22.17	21.77	21.89	22.22
B08	31.6	30.12	31.96	32.58	32.3	32.64	31.81	32.56	32.99	32.67	33.46	32.36	33.19	32.07	32.68	32.5	33.23	32.91	33.55	31.46	31.46	31.53	32.12
B09	26.23	24.81	24.78	24.53	24.87	24.88	25.49	24.91	25.01	24.49	24.85	25.02	24.88	24.92	26.68	25.51	25.46	25.11	25.94	25	24.62	24.72	24.82
B10	35	35.94	35	35	35	38.33	35	35	35	35	35	37.73	35	35	35	35	35	36.78	35	35	38.15	35	35
B11	35	34.99	35.02	32.54	34.52	30.59	29.89	31.2	33.36	33.35	33.05	35	33.6	33.56	32.96	35.17	33.4	33.81	35.18	34.74	32.81	33.88	35.53

B12	33.93	35.29	32.61	31.76	32.15	31.76	32.41	33.31	32.21	30.79	32.28	32.81	32.02	32.78	36.19	34.53	32.47	32.25	33.08	31.16	31.97	31.58	34.43
C01	24.28	25.39	23.83	23.63	24.01	23.88	24.31	23.64	24.14	23.43	23.86	24.13	24.08	24.03	24.71	24.08	24.11	24.23	24.91	24.27	23.81	24.01	24.22
C02	24.79	24.7	24.28	24.25	24.26	24.2	24.91	24.19	24.7	23.95	24.69	24.73	24.55	24.76	25.87	24.64	24.75	24.79	25.79	24.39	24.44	24.52	24.77
C03	23.74	23.85	23.42	23.36	23.49	23.47	23.93	23.38	23.64	23.13	23.55	23.72	23.8	23.82	25.32	24.16	23.88	23.89	24.67	23.69	23.57	23.59	23.85
C04	22.59	28.69	20.14	19.77	20.54	21.56	22.06	21.24	20.79	20.18	20.4	20.64	20.41	20.78	22.82	21.11	21.12	20.64	21.04	20.69	20.07	20.32	20.22
C05	22.81	24.25	22.67	22.62	22.5	22.57	22.78	22.35	22.91	22.25	22.77	22.81	22.77	22.83	24.63	22.89	22.93	23.07	23.74	22.84	22.63	22.74	22.87
C06	26.43	26.6	26.62	26.84	26.7	26.63	26.97	26.59	26.98	26.16	26.89	26.9	26.77	26.76	27.88	27.02	26.81	27.22	27.83	26.75	26.69	26.66	27.03
C07	22.28	22.88	21.19	21.37	21.46	21.53	21.62	21.63	21.71	21.61	21.73	21.66	22.08	21.87	23.07	22.14	22.17	22.31	22.86	20.7	20.58	20.63	20.93
C08	33.52	35	32.85	33.68	33.41	33.33	32.55	33.72	32.8	32.13	32.46	33.39	32.58	33.26	35	34.22	33.2	35.08	33.49	34.04	33.51	33.18	35.2
C09	26.1	25.88	25.67	25.78	25.68	25.6	25.83	25.54	25.95	25.35	25.84	25.84	25.88	25.73	27.45	26.01	26.07	26.16	26.84	25.78	25.73	25.72	26.06
C10	25.68	24.03	25.02	25.24	25.58	25.02	25.55	24.98	25.6	24.57	25.34	25.15	24.99	25.23	26.49	25.83	25.4	25.54	26.26	25.74	25.01	25	25.65
C11	24.73	23.39	24.63	24.03	26.06	24.36	24.84	24.63	24.82	23.39	24.89	24.83	24.6	25.24	27.43	26.56	24.72	24.94	26.03	24.25	25.61	24.34	27.35
C12	26.91	26.42	26.62	26.3	26.89	26.23	26.49	26.41	27.14	25.85	27.3	26.69	26.76	26.74	28.04	27.91	27.15	27.57	27.68	26.35	26.75	26.89	27.71
D01	26.53	30.92	24.45	24.04	24.68	25.41	25.91	25.54	25.01	24.66	24.64	24.97	24.86	25.03	26.85	25.65	25.68	25.28	25.71	24.9	24.63	24.71	24.75
D02	25.62	24.98	25.47	25.72	25.17	24.91	25.63	25.1	25.55	24.85	25.8	25.49	25.63	25.35	27.7	25.48	25.43	25.67	26.6	25.29	25.15	25.31	25.8
D03	24.99	28.51	25.55	25.3	25.06	24.92	25.56	25.16	25.67	24.58	25.7	25.58	25.16	25.14	28.77	25.24	25.11	25.25	26.19	25.27	24.89	24.9	25.31
D04	23.89	22.53	23.04	23.12	23.08	23.17	23.57	23.08	23.49	23.03	23.5	23.44	23.5	23.6	24.71	23.55	23.75	23.76	24.61	22.89	22.85	22.95	23.23
D05	22.79	23.95	23.71	23.67	23.43	23.09	23.4	23.1	23.75	23.1	23.78	23.73	23.81	23.72	25.74	23.89	23.74	23.93	24.72	23.92	23.57	23.59	23.97
D06	35	34.23	35	36.17	34.65	33.99	34.5	35.07	35.26	36.24	34.13	34.47	35.67	34.16	36.38	35.41	34.92	35.63	35.61	34.71	34.15	33.26	33.14
D07	22.36	21.87	22.52	22.53	22.62	22.46	22.89	22.52	22.82	22.24	22.83	22.82	22.92	22.92	24.63	23.42	23.04	22.99	24	23.24	22.85	22.8	23.56
D08	26.79	26.14	26.57	26.75	26.61	26.13	26.34	26.42	26.83	26.2	27.01	26.65	26.91	26.57	27.19	26.84	27.12	26.96	27.61	26.12	25.82	25.87	26.46
D09	26.34	26.99	25.72	25.45	26.24	25.71	26.3	25.69	25.93	25.22	25.91	25.86	25.87	26.19	27.93	26.43	26.04	26.05	26.8	26.65	25.99	25.89	26.77
D10	22.14	23.19	21.95	21.78	21.69	21.33	21.78	21.4	22.03	21.23	21.88	21.88	21.91	21.83	23.29	21.84	22.02	22.07	22.79	21.84	21.65	21.69	22.05
D11	23.25	24.14	22.77	22.94	23.02	22.72	22.99	22.73	23.31	22.6	22.98	22.91	23.16	23.15	24.28	23.42	23.35	23.72	24.55	22.82	22.67	22.67	23.02
D12	28.21	26.81	26.68	26.75	27.23	26.91	27.41	26.96	27.16	26.62	26.97	27.15	27.23	27.59	28.65	27.75	27.57	27.36	28.09	26.79	27.15	26.97	27.64
E01	34.04	35	32.44	32.59	32.06	32.47	33.74	32.61	33.34	32.05	33.3	32.95	33.05	32.97	34.81	34.73	32.86	32.77	33.42	32.31	32.58	33.2	33.02
E02	24.2	24.45	24.13	23.8	23.92	23.86	24.22	23.74	24.23	23.66	24.13	24.22	24.24	24.31	26.26	24.28	24.4	24.39	25.36	24.33	24.09	24.18	24.46
E03	23.33	24.35	23.77	23.71	23.61	23.81	24.07	24.17	24.43	23.83	24.31	24.18	24.32	23.97	25.93	24.23	24.51	24.58	25.19	23.04	23.17	23.22	23.47
E04	25.25	26.31	23.64	23.53	23.82	23.86	24.11	23.96	24.08	23.64	23.9	24.05	24.13	24.02	25.77	24.45	24.49	24.24	25.06	23.69	23.66	23.67	23.8

E05	25.88	26.75	25.82	25.83	25.8	25.67	26.06	25.83	26.03	25.62	26.08	26.03	26.18	26.04	27.15	26.37	26.39	26.44	27.04	25.64	25.27	25.46	25.75
E06	32.91	33.78	30.22	29.52	30.29	30.47	31.04	30.28	30.56	30.04	30.26	30.3	30.02	30.76	32.13	30.78	30.66	30.49	31.23	30.37	30.58	30.02	30.7
E07	29.27	30.05	29.29	29.3	29.68	28.86	29.16	28.75	29.5	28.83	29.44	29.59	29.47	29.65	29.36	29.92	29.8	29.9	31.06	29.96	29.26	29.71	29.95
E08	25.99	24.72	24.06	24.36	24.01	23.86	24.22	24.29	24.42	23.58	24.35	24.33	24.34	23.66	26.41	24.16	24.38	24.26	24.89	23.42	22.85	23.08	23.57
E09	25.49	25.42	24.64	24.57	24.85	24.42	24.72	24.21	24.86	23.87	24.59	24.73	24.42	24.78	26.34	25.09	24.78	24.89	25.39	25.2	24.76	24.71	25.36
E10	28.33	30.09	27.78	27.4	28.17	27.66	27.82	27.53	28.02	27.29	27.75	28	28.15	28.51	28.12	28.83	28.17	28.21	28.74	29.06	28.4	28.37	28.82
E11	25.36	25.37	25.08	24.93	25.26	24.91	25.44	24.75	25.46	24.41	25.1	25.35	25.24	25.39	26.83	25.67	25.23	25.18	26.06	26.13	25.29	25.25	26.34
E12	22.48	23.91	23.78	23.75	23.72	23.32	23.83	23.57	24.06	23.14	24.03	24.35	24.03	24.16	26.19	24.21	23.97	24.35	25.03	23.72	24.23	23.95	24.98
F01	26.49	31.81	29.47	28.95	28.15	27.49	27.03	27.57	29.25	27.98	29	29.87	29.12	28.78	31.94	29.65	28.72	29.29	29.97	29.04	29.17	28.96	29.59
F02	36.01	36.89	35	35	36.57	33.57	34.54	33.87	34.88	36.42	35.17	37.29	35.41	35.6	36.06	35	35.75	35.16	38.2	35.13	36.92	33.91	37.42
F03	26.14	24.62	25	24.79	25.33	25.05	25.61	24.85	25.05	24.7	25.17	25.41	25.35	25.47	26.85	25.75	25.4	25.41	26.2	25.54	25.17	25.2	25.73
F04	25.47	24.82	24.79	24.6	25.09	25	25.23	24.87	25.13	24.66	24.88	25.12	25.07	25.23	26.87	25.42	25.47	25.32	25.95	25.34	24.8	24.84	25.17
F05	24.24	23.89	23.5	23.36	23.39	23.36	23.77	23.48	23.71	23.04	23.62	23.62	23.64	23.69	24.81	23.78	24	23.85	24.71	23.61	23.28	23.41	23.59
F06	22.71	24.06	22.91	23.07	22.85	22.69	23.26	22.82	23.31	22.62	23.2	23.22	23.28	23.25	24.74	23.65	23.44	23.61	24.31	23.45	23.14	23.03	23.61
F07	25.08	25.45	24.87	24.82	25.01	25.14	25.37	24.91	25.41	24.85	25.16	25.32	25.34	25.37	26.35	25.66	25.7	25.73	26.54	25.44	25.29	25.49	25.58
F08	23.8	23.58	23.59	23.59	23.71	23.61	23.87	23.43	23.88	23.46	23.79	23.87	23.88	24.08	25.05	24.25	24.24	24.2	24.87	24.32	24.12	24.16	24.51
F09	34.83	33.67	33.31	33	35.54	34.32	36.32	34.47	35.75	33.66	33.91	36.11	34.17	35.28	35	35.65	34.64	34.14	34.73	35.83	32.25	32.4	33.84
F10	23.99	26.3	22.78	22.77	22.77	21.69	22.34	21.49	22.37	21.54	22.77	22.66	22.31	22.58	26.29	22.58	22.09	22.35	23.43	23.12	22.71	22.37	23.39
F11	19.7	21.26	20.17	20.28	19.8	20.03	20.33	20.19	20.61	19.78	20.38	20.38	20.36	20.34	23.4	20.71	20.57	20.75	21.18	20.64	20.46	20.34	20.84
F12	18.2	20.65	18.89	19.06	18.69	18.35	18.88	18.56	19.03	18.18	19.27	18.74	18.91	18.84	21.58	18.97	18.72	19.07	19.78	18.64	18.79	18.69	19.54
G01	17.57	18.5	21.33	21.02	20.02	19.93	20.34	20.33	21.24	20.22	21.22	21.41	21.08	20.87	21.01	21.29	21.14	21.54	22.32	20.85	20.91	20.78	21.15
G02	27.88	27.59	27.71	27.91	28.31	28.01	28.74	28.01	28.15	28.18	28.52	28.46	28.84	28.65	29.75	29.33	28.66	28.71	29.76	28.49	28.57	28.44	28.67
G03	30.5	32.54	29.65	29.34	29.75	29.55	29.79	29.08	29.66	28.98	29.62	29.99	29.79	29.65	31.39	30.04	29.89	29.83	30.5	29.97	29.7	29.61	30.11
G04	35	35	35	35	34.71	35	35.92	35	35	35	35	37.46	35	38.44	35	35	35	35	35	37.97	35	35	35
G05	25.76	24.73	25.51	25.29	25.4	25.36	25.69	25.17	25.82	25.09	25.76	25.68	25.76	25.75	26.82	25.98	26	26.03	26.83	25.96	25.88	25.94	26.2
G06	26.48	28.55	25.64	25.76	25.91	25.68	25.81	25.97	26.19	25.71	26.07	26.21	26.29	25.93	27.91	26.38	26.71	26.56	27.05	25	24.92	24.97	25.21
G07	29.64	30.41	29.32	29.61	29.04	35	29.65	28.98	29.03	28.9	29.24	29.8	29.69	29.32	30.85	29.61	29.89	29.48	30.58	29.09	28.49	28.68	29.16
G08	34.41	37.06	30.64	30.51	32.04	32.83	33.51	32.72	31.68	31.41	31.09	31.42	31.15	31.62	33.49	32.91	33.09	31.57	31.99	32.12	30.83	31.02	30.87
G09	24.66	24.26	24.43	24.58	24.63	24.32	24.65	24.16	24.78	24.17	24.66	24.74	24.79	24.81	26.58	24.92	24.88	25.01	25.81	24.98	24.85	24.9	25.42

G10	22.58	20.66	21.91	22.02	22	21.96	21.97	21.93	22.44	21.86	22.43	22.38	22.56	22.26	23.06	22.86	22.85	22.86	23.41	22.36	22.23	22.17	22.63
G11	25.55	24.74	24.86	24.83	24.99	25.01	25.5	24.99	25.38	24.64	25.17	25.4	25.23	25.4	27.26	25.5	25.52	25.56	26.06	25.55	25.07	24.94	25.67
G12	27.43	26.06	26.89	27.12	27.26	26.75	27.09	26.91	27.22	26.61	27.33	26.84	27.34	27.2	27.97	27.44	27.37	27.41	28.46	26.71	26.87	26.79	27.36
H01	17.29	18.44	17.62	17.62	17.52	17.08	17.6	17.33	17.75	16.83	17.7	17.68	17.45	17.58	19.19	18.03	17.78	17.79	18.62	17.74	17.49	17.49	17.96
H02	19.14	19.31	18.87	18.69	19.21	18.8	19.4	18.67	19.17	18.43	19.11	19.12	18.94	19.17	21.3	19.34	19.2	19.03	20.07	19.17	19.01	19.14	19.35
H03	18.8	21.02	19.79	19.82	19.11	18.96	19.42	19.31	19.81	18.92	19.93	19.76	19.62	19.45	21.66	19.63	19.65	19.83	20.4	19.17	19.13	19.06	19.58
H04	25.53	25.39	25.45	25.28	25.26	25.36	25.8	25.4	25.72	25.17	25.6	25.68	25.74	25.75	26.78	25.8	25.92	25.88	26.67	25.75	25.61	25.68	25.91
H05	18.61	18.16	18.24	18.07	18.22	18.03	18.48	17.78	18.3	17.61	18.27	18.3	18.19	18.44	20.04	18.53	18.25	18.35	19.02	18.85	18.15	18.23	18.69
H06	35	37.94	37.61	35	35	35	35	35	34.25	35	35.91	34.84	35.81	35	35	36.08	35	35	35	35.98	35	34.72	35
H07	22.93	22.95	23.11	23.43	23.43	22.88	23.22	23.15	23.04	22.84	23.19	23.35	23.21	23.06	23.55	23.8	23.34	23.21	23.79	22.89	22.58	22.45	23.1
H08	23.07	22.99	23.26	23.44	23.34	22.96	23.2	23.13	23.02	22.85	23.09	23.28	23.27	23.1	23.48	23.81	23.16	23.18	23.87	23.07	22.6	22.57	23.02
H09	23.15	23.08	23.49	23.6	23.6	23.17	23.58	23.21	23.25	22.98	23.28	23.38	23.38	23.32	23.62	23.96	23.2	23.33	23.82	23.32	22.73	22.55	23.28
H10	19.71	19.55	19.77	19.66	20.35	19.61	19.89	19.95	19.93	19.51	19.91	19.87	19.95	19.93	20.15	20.61	19.68	19.73	19.96	20.96	20.08	19.57	20.68
H11	19.39	19.58	19.74	19.56	20.12	19.64	19.91	19.88	19.82	19.43	19.81	19.88	19.89	19.93	20	20.55	19.71	19.86	19.87	20.93	19.86	19.54	20.65
H12	19.46	19.6	19.79	19.49	20.33	19.62	19.87	19.9	19.8	19.57	19.74	19.65	19.88	19.92	20.21	20.47	19.72	19.95	19.96	22.26	20.12	19.59	20.58